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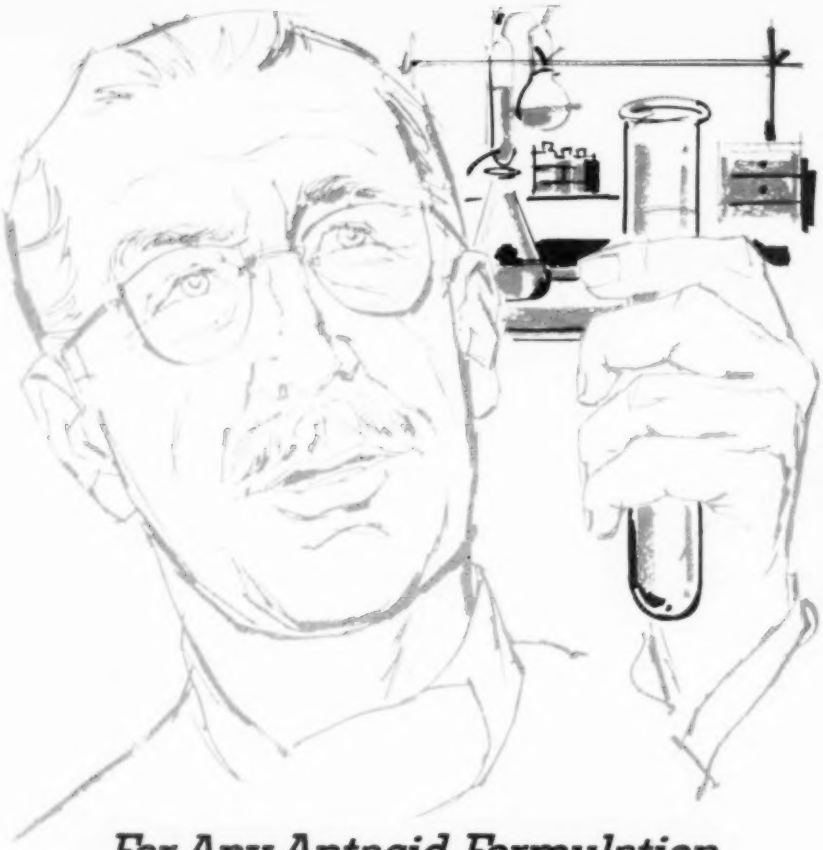
Investigation of Drug Release from Solids III. Effect of a Changing Surface-Weight Ratio on the Dissolution Rate 335
Dale E. Wurster and *James A. Seitz*
The Effect of Surfactants on Drug Stability I 339
S. Riegelman
A New Method of Manufacture of Tablet Granulations I 344
P. A. Tuerck, E. L. Walters, and *E. D. Carkhuff*
A New Method of Manufacture of Tablet Granulations II 347
P. A. Tuerck, E. L. Walters, and *E. D. Carkhuff*
Esterification of Two Sterically Hindered Acids Using Ultrasound Waves 350
Robert J. Gerraughty and *Paul J. Jannke*
Studies on the Stability of Filipin I. Thermal Degradation in the Presence of Air 352
James E. Tingstad and *Edward R. Garrett*
The Complexing Tendencies of Cyanocobalamin With Inorganic Compounds. Heteromolybdates and Heavy Metal Chlorides 356
Ruth N. Havemeyer and *Takeru Higuchi*
Antioxidants. The Microdetermination of Hydroquinone 360
Mohamed Z. Barakat, Saad K. Shehab, and *Abdel Maksoud Abdalla*
Isomerization of Vitamin A in Aqueous Multivitamin Drop Preparations 363
Robert W. Lehman, John M. Dieterle, William T. Fisher, and *Stanley R. Ames*
Estimation of the Biological Potency of Isomerized Vitamin A Palmitate in Aqueous Multivitamin Dispersions from Maleic Values 366
Stanley R. Ames, William J. Swanson, and *Robert W. Lehman*
The Isolation of β-Allocryptopine from *Argemone squarrosa* subsp. *squarrosa* 368
T. O. Soine and *R. E. Willette*
The Application of Alternating Current Polarography in the Determination of Phenobarbital in Dosage Forms 371
N. G. Lordi, E. M. Cohen, and *B. L. Taylor*
Study of the Stability of Secobarbital Sodium Solutions I. Determination of Solubility of Secobarbital as a Function of Solvent and Hydrogen Ion Concentration 376
J. H. Udani and *J. Autian*
Study of the Stability of Secobarbital Sodium Solutions II. Separation and Identification of Degradation Products of Secobarbital Sodium 380
A. J. Kapadia and *J. Autian*
The Hydrolysis of Hydrocortisone Phosphate in Essentially Neutral Solutions 383
Arnold D. Marcus
The Photometric Determination of Hydrochlorothiazide and Its Hydrolysis Product 386
C. R. Rehm and *J. B. Smith*
The Metabolism and Central Nervous System Distribution of C-14 Carbonyl Salicylamide in the Rat 389
William F. Bousquet and *John E. Christian*
Studies on Local Anesthetics XXV (Physicochemical Properties of Drugs V) 394
Aleš Sekera and *Čeněk Vrba*
The Design of a Continuous Recording *In Vivo* Method of Measuring Sensible Perspiration Over a Limited Area 398
William J. O'Malley and *John E. Christian*
An Evaluation of the Ability of Antiperspirant Compounds to Reduce Perspiration Flow 402
William J. O'Malley and *John E. Christian*
Determination of Vitamin A: Errata 404
E. G. E. Shafer
A Note on Esters of β-Phenyl-α-benzyloximinopropionic Acid 405
Louis Gass and *John W. Martin, Jr.*
A Note on the Synthesis of C-14 Carboxyl Salicylic Acid by the Halogen-Metal Interconversion Reaction 406
William F. Bousquet and *John E. Christian*
A Note on Improvement of Separations in Paper Partition Chromatography 408
Jere E. Goyan
Book Notices 409
The United States Pharmacopoeial Convention, Inc. Annual Financial Statement 410
Advertising ..... Cover pages 2, 3, and 4, and I to IV



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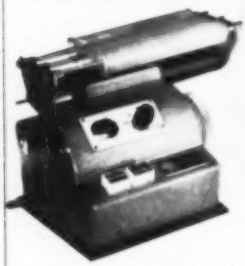

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JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

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Investigation of Drug Release from Solids III*

Effect of a Changing Surface-Weight Ratio on the Dissolution Rate

By DALE E. WURSTER and JAMES A. SEITZ†

A dissolution study was conducted in which the surface area-weight ratio of a dissolving solid was not held constant. Cylindrical tablets containing large pores were employed to follow the dissolution process. Studies in distilled water indicated that the surface area of the tablet pore was incompletely exposed to the solvent due to occlusion by air. Solutions with a lower surface tension than water were capable of wetting the entire surface area and a corresponding increase in the dissolution rate was obtained. When the air was evacuated from the artificially produced tablet pores the solvent was permitted to come in contact with the entire surface and the dissolution rate increased. Dissolution from the pore surface occurred at a slower rate than from the exterior surface due to the longer diffusional pathway of the solute molecules.

THE FACTORS influencing the dissolution rate of a slightly-soluble medicinal agent are many and varied but possibly none of them play a more important role than the surface characteristics (1–3) of the solid particle.

The dissolution behavior of materials obtained from granulation, lyophilization, and other procedures would be especially difficult to predict due to the variation in fissures and pores introduced into the solids during the drying stages. Since the dissolution rate is proportional to the surface area of the dissolving particle, measurements of the total surface area as well as the area calculated from the gross particle size might lead to erroneous calculations of the rate in a diffusion-controlled process.

The present investigation was, therefore, undertaken to study the factors influencing dissolution of slightly-soluble solids in which the surfaces are of a varying degree of availability to the solvent.

* Received April 25, 1958, from the University of Wisconsin, School of Pharmacy, Madison 6.

This paper is based on a dissertation submitted by James A. Seitz to the Graduate School of the University of Wisconsin in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

† University of Wisconsin fellow.

Presented to the Scientific Section, A. PH. A., Los Angeles meeting, April 1958.

METHOD OF STUDY

Previous workers (4–6) have generally circumvented the problem of a changing surface either by maintaining it relatively constant or by using geometric shapes where the surface could be readily calculated during the dissolution process. Equations describing dissolution kinetics show that the dissolution rate is directly proportional to the area exposed to the solvent under conditions of uniform agitation and a constant shape. Therefore, particles with a highly irregular or fissured surface should have a faster overall rate of solution, if the entire surface is exposed to the solvent, than spherical particles where the surface area per unit weight is minimal.

In order to study the availability of the surface to the solvent, model porous particles were prepared by introducing artificial pores into cylindrical compressed tablets. The initiating work of Parrott (5) indicated that the porosity of an isotropic crystalline mass such as that in compressed tablets was of sufficiently low magnitude that the surface-weight ratio remained constant and the tablet could be treated as a homogeneous solid. However, in the case where the pores are sufficiently large or the conditions such that the pore surface participates in the dissolution process, a varying surface-weight ratio results. The availability of the pore surface was studied by following the dissolution rate of cylindrical benzoic acid tablets in distilled water, in a sodium lauryl sulfate solution, and gas-evacuated tablets in distilled water.

The mathematical interpretation is based on the Hixson-Crowell (7, 8) equation

$$dw/dt = -3KS = -3\,Ka\,W^{2/3} \quad \text{(Eq. 1)}$$

which upon integration yields

$$Kat = W_0^{1/3} - W^{1/3} \quad \text{(Eq. 2)}$$

where K is the velocity constant, S is surface area, a is the surface-weight constant, t is time, and W_0 and W are the weights at $t = 0$ and t, respectively. The constant, a, for a cylinder or sphere, where the shape remains constant throughout the dissolution study, can be determined mathematically or graphically as $S = a\,W^{2/3}$. When the surface-weight ratio is not constant, as in the case of solids with pores large enough to be involved in the dissolution process, it is possible to use a reduced form of Eq. 2,

$$K't = W_0^{1/3} - W^{1/3} \quad \text{(Eq. 3)}$$

where K' is comparable to the constants Ka. Experimental evidence indicates K' to be constant during the initial phase of the dissolution process. The velocity constant K during this time period can be evaluated by determining the surface-weight ratio at the same time.

EXPERIMENTAL

Procedure.—The experimental procedure of Parrott (5) was followed except for minor alterations. During the dissolution procedure the tablet was removed from the solution at regular time intervals, rapidly dried, and the dimensions measured with an optic micrometer (Gaertner Scientific Corp.) on a rotating substage. The depth of the artificial pores was also determined. The diameter at the base of the pore was calculated by assuming the change in the depth of the pore was equal to the change in the diameter at the base. The dimensions were measured to 0.0001 cm., with the number of readings varying from 24 for the solid tablets to 84 for the 6-pore tablets. After weighing, the tablet was returned to a fresh 2,000-ml. quantity of solution for another cycle.

The 0.2% sodium lauryl sulfate solutions were prepared with freshly-boiled distilled water and then neutralized to phenol red indicator with 0.02 *N* HCl.

The gas-evacuated tablets were evacuated for not less than two hours with a Cenco-Pressovac vacuum pump. The tablet evacuation chamber was a modified $^{19}/_{38}$ ground-glass joint with ground-glass stopcocks on either end. One stopcock opened to the vacuum pump and the other to the distilled water flask. A flask containing freshly-boiled distilled water was attached to the chamber while the water was still vaporizing to insure that no air would be absorbed on cooling. After the water cooled to the desired temperature it was allowed to envelop the tablet, and the contents of the chamber were immediately transferred to the agitated liquid in the dissolution vessel.

The tablets were compressed with an instrumented Stokes (model A-3) single punch tablet machine equipped with $^{3}/_{8}$ inch flat punches. The tablets were all compressed with the same compressional force as recorded by oscillograph recordings. The artificial pores were introduced by drilling holes in the flat surfaces of the tablets with a drill press using a 0.04-inch diameter drill. These holes of uniform depth were placed equidistant between the center and the edge of the tablet.

Using the procedures outlined above, the dissolution behavior of solid cylindrical benzoic acid tablets and tablets containing 1 to 6 large pores was investigated. The rate of solution of these tablets was studied in distilled water and 0.2% sodium lauryl sulfate solution. The dissolution rate of air-evacuated tablets in distilled water is also reported.

Reagents.—Benzoic acid, reagent grade; sodium lauryl sulfate U. S. P.

RESULTS AND DISCUSSION

Dissolution Study in Distilled Water.—In the derivation of Eq. 3 it was assumed K' would be constant for the initial phase of the dissolution process. When $W_0^{1/3} - W^{1/3}$ was plotted against time a straight line relationship was obtained which indicated this assumption to be correct. However, the same linearity would be obtained if the pores were occluded by air or other adsorbed gases. Likewise, the surface-weight ratio, a, and the velocity constant, K, would then be constant as observed for solid cylindrical tablets. Although the Ka values were generally increasing as more pores were introduced into the tablets, this increase was due largely to the surface-weight factor, a.

In evaluating the constant, a, an indication of the degree of participation of the internal pore surface was obtained when the experimental line was compared with a theoretical line, where it was assumed that the pore surface did not participate in the dissolution process. It can be seen in Fig. 1 that there was practically no difference between the experimental and the theoretical lines.

As shown in Table I, the rate of dissolution ($3KS$) of the pore-containing tablets increased, but at a much slower rate than might be expected. With the introduction of six pores the surface area was increased over 20%, but the increase in the $3KS$ value was less than 4%. Since the external surface accounted for about 96% of the benzoic acid dissolved, this suggests that the pores were largely occluded by air and the area was unavailable to the solvent. Pore diameter measurements supported this for only the orifices of the pores exhibited any enlargement during the dissolution study.

Dissolution Studies in 0.2% Sodium Lauryl Sulfate Solution.—To study the effect of a lowered surface tension on pore penetration and the dissolution rate, a 0.2% aqueous sodium lauryl sulfate solution was used. As in the case of distilled water the Ka value increased as more pores were introduced into the tablet, but unlike the previous study the surface-weight factor, a, was lower. In Fig. 2 the participation of the pores in the dissolution process can readily be seen when the experimental line is again compared with the previously mentioned theoretical line. During the dissolution process the pores became considerably enlarged. The dissolution from the cylindrical pores was not uniform but varied with the distance from the pore orifice, and at the completion of the dissolution study the shape of the pores was conical.

This alteration in the shape was theoretically expected due to the longer diffusional pathway of the solute molecules and the decreased agitation of the solvent in the pores which results in a progressively slower dissolution rate as the depth into the pore increases. It is obvious that the evaluation of K for porous solutes is related to the rate of change of the surface-weight factor, a. If the a value decreases and at the same time the Ka value increases, the velocity constant, K, will be greater and an increase in the rate of dissolution ($3\,KS$) (Table II) results.

Dissolution Study of Gas-Evacuated Tablets in Distilled Water.—When the tablets were gas evacuated the solvent was able to penetrate the pores and wet the entire surface. The result of gas evacuation was comparable to that observed by lowering the surface tension of the solvent (Fig. 2 and Table III). Visually, it was observed that the pores enlarged conically in a manner analogous to that with the sodium lauryl sulfate solution which, again, can be explained on the basis of the decreased dissolution rate in the pores resulting from the increased diffusional pathway of the solute molecules.

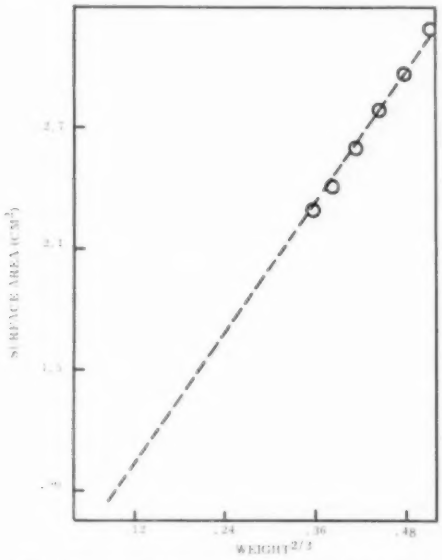

Fig. 1.—Comparison of theoretical with experimental surface-weight factor values for dissolution in distilled water. – – – Theoretical line based on only the external surface of a 6-pore tablet participating in the dissolution process; ○ experimental values for a 6-pore tablet.

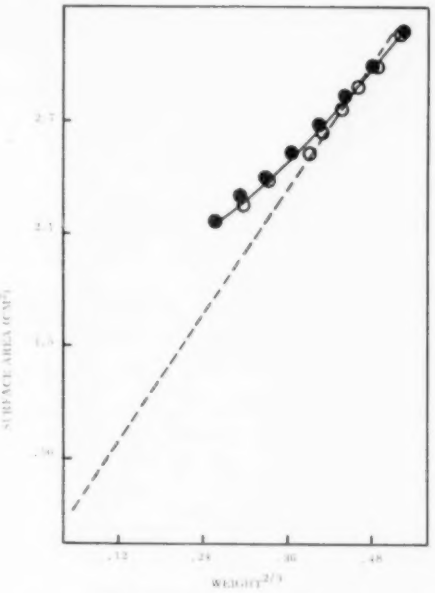

Fig. 2.—Comparison of theoretical with experimental surface-weight factor values. – – – Theoretical line based on only the external surface of a 6 pore tablet participating in the dissolution process; —— experimental line; ○ 6-pore tablet in 0.2% sodium lauryl sulfate solution; ● 6-pore air-evacuated tablet in distilled water.

In the graphic determination of the Ka values ($W_0^{1/3} - W^{1/3}$ *vs.* time) for the pore-containing gas-evacuated tablets a very slight departure from linearity occurred after one hour of dissolution. However, in all other cases, a straight line relationship was maintained throughout the entire required dissolution period one and one-half to two hours.

In all cases, with the introduction of pores into tablets the Ka values increased over those obtained for solid tablets but the increase varied with the experimental conditions. In sodium lauryl sulfate solutions the increase in the Ka values was the greatest, and in distilled water it was the least (Fig. 3). The difference in the Ka values for gas-

TABLE I.—COMPARISON OF DISSOLUTION RATES OF TABLETS WITH VARYING SURFACE AREAS IN DISTILLED WATER

Tablets	Ka	a	K	Total Surface Area, S, cm.²	Surface Area of Pores cm.²	$3\,KS$, Gm./cm.²/hr.
Solid	0.0903	4.53	0.0199	2.491	. . .	0.1487
1 Pore	0.0987	4.76	0.0188	2.634	0.075	0.1486
2 Pores	0.0924	4.85	0.0191	2.693	0.186	0.1543
3 Pores	0.0924	5.21	0.0177	2.754	0.276	0.1462
4 Pores	0.0920	5.18	0.0178	2.849	0.371	0.1521
5 Pores	0.0960	5.68	0.0169	2.941	0.469	0.1491
6 Pores	0.0951	5.57	0.0171	3.004	0.564	0.1541

TABLE II.—COMPARISON OF DISSOLUTION RATES OF TABLETS WITH VARYING SURFACE AREAS IN 0.2% SODIUM LAURYL SULFATE SOLUTION

Tablets	Ka	a	K	Total Surface Area, S, cm.²	Surface Area of Pores cm.²	$3KS$, Gm./cm.²/hr.
Solid	0.0961	4.57	0.0210	2.552	...	0.1608
1 Pore	0.1039	4.63	0.0224	2.647	0.101	0.1779
2 Pores	0.1090	4.28	0.0255	2.697	0.189	0.2063
3 Pores	0.1118	4.19	0.0267	2.798	0.291	0.2241
4 Pores	0.1158	4.43	0.0262	2.869	0.373	0.2255
5 Pores	0.1187	4.15	0.0286	2.944	0.460	0.2526
6 Pores	0.1207	4.44	0.0272	3.022	0.530	0.2466

TABLE III.—COMPARISON OF DISSOLUTION RATES OF AIR-EVACUATED TABLETS WITH VARYING SURFACE AREAS IN DISTILLED WATER

Tablets	Ka	a	K	Total Surface Area, S, cm.²	Surface Area of Pores, cm.²	$3KS$, Gm./cm.²/hr.
Solid[a]	0.0903	4.53	0.0199	2.491	...	0.1487
2 Pores	0.0986	4.50	0.0219	2.671	0.183	0.1754
4 Pores	0.1043	4.60	0.0226	2.870	0.388	0.1946
6 Pores	0.1121	4.05	0.0277	3.008	0.563	0.2500

[a] Solid tablets were not air evacuated.

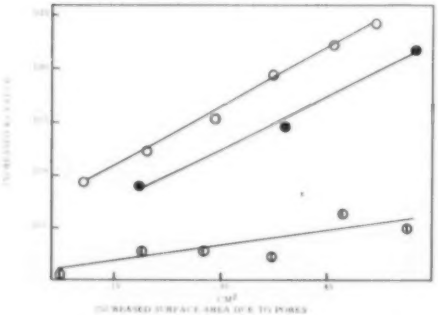

Fig. 3.—Effect of increased "effective" surface area on the Ka values. ○ 0.2% sodium lauryl sulfate solution; ● air-evacuated tablets in distilled water; ① distilled water.

evacuated tablets and tablets dissolved in 0.2% sodium lauryl sulfate solution can be attributed to the greater solubility of benzoic acid in the sodium lauryl sulfate solution. This increased solubility was substantiated by separate solubility tests.

A subsequent paper will deal with the dissolution behavior of lyophilized solids.

SUMMARY

The dissolution rate of benzoic acid tablets with varying surface areas was investigated in distilled water and 0.2% sodium lauryl sulfate solution. The dissolution process of air-evacuated tablets in distilled water was also investigated.

In distilled water the dissolution study indicated that pores in the benzoic acid tablets were somewhat occluded by air and, therefore, this surface area was not entirely available to the solvent.

The entire surface area of the pore-containing benzoic acid tablets participated in the dissolution process when 0.2% sodium lauryl sulfate was used. The increased dissolution rate was probably due to the lower surface tension, allowing penetration of the pores by the solvent.

Air evacuation of the benzoic acid tablets permitted the water to enter the pores. Results obtained were analogous to those obtained with 0.2% sodium lauryl sulfate solution.

The availability of the internal surface of porous particles to the solvent action is important in evaluating the effect of an increased surface area on the dissolution rate.

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The Effect of Surfactants on Drug Stability I*

By S. RIEGELMAN

The effect of surfactants on the rate of hydrolysis of esters, using benzocaine as an example, is reported for a homologous series of alkali-stable nonionic surfactants, and with cationic and anionic surfactants. It was found that the hydrolysis rate of the ester in the alkali-stable nonionic surfactants varies with the concentration of the surfactant more than with the length of the polyoxyethylene chain of the surfactant. Hydrolysis apparently takes place within the micelle, as well as in the aqueous phase. Anionic and cationic surfactants appear to stabilize the drug to base catalysis, resulting in an eighteen-fold increase in half-life in 5 per cent lauryl sulfate solutions. A dilute solution of the quaternary, cetyl trimethyl ammonium bromide, slightly accelerates the rate of hydrolysis.

THE MICELLULAR AGGREGATES of surfactants possess the well-known property of increasing the solubility of water-insoluble organic subsstances. Many studies have been made, particularly with the nonionic group of surfactants, as to their usefulness as solubilizing agents for antibacterial agents (1–5), oil-soluble vitamins (6–8), steroids (9), estrogens (10), and for other substances. Several studies have been undertaken to clarify the effect solubilization has on drug availability. These include the release of phenols (2, 3), iodine (11), parabens (4, 5), and estrogens (12). Little is known, however, of the effect solubilization will have on the stability of a drug to hydrolytic attack. The solubilized drug may be held deep within the hydrocarbon interior of the micelle. Under these conditions, it is probable that the hydroxyl or hydrogen ions will not be able to make contact with the solubilized drug, and no hydrolytic cleavage will result. Solubilization may take place by other mechanisms, i. e., surface adsorption and short or deep penetration of the palisade layers of the micelle. Under the latter conditions, acid or base catalysis may or may not be able to take place. If the surface of the micelle is highly hydrated, nonionic surfactants may allow the catalytic acidic or basic ion to penetrate and to make contact with the solubilized drug within the polyoxyethylene chains. The length of the polyoxyethylene chain, the concentration of the surfactant and of the drug should all influence the rate of hydrolysis under standard conditions. Micelles of charged surfactants may have a different effect on the hydrolysis rate, since coulombic forces now play a predominant role in determining whether the catalytic ion may be able to invade the micelle to reach the solubilized drug. In all of the cases, the amount of drug in the free aqueous phase will undergo base or acid catalysis and in many cases may be the only site of hydrolysis.

Benzocaine was selected as the ester chosen for study in this preliminary report. Higuchi and Lachman (13) carried out a study of the base catalyzed hydrolysis of benzocaine in water in a complex with caffeine. The presence of 2.5 per cent of caffeine in the system resulted in reduction of the hydrolytic rate to less than one-fifth of that in the absence of the caffeine. Their results indicated that the complexed form of the drug does not undergo any perceptible cleavage of the ester linkage.

The present study will show that the use of solubilization surfactants to solubilize a major portion of a drug can result in as much as an eighteen-fold stabilization of the drug to hydrolysis. Evidence is gathered which indicates that the drug undergoes base catalysis even within the nonionic surfactant micelle.

EXPERIMENTAL

Reagents.—*Benzocaine.*—U. S. P. product, recrystallized from ethyl alcohol and water, m. p. 92–93°.

Sodium Lauryl Sulfate.—A U. S. P. grade (Dupanol C, DuPont de Nemours) was recrystallized from hot absolute ethyl alcohol and washed with ether. The compound was substantially free from extraneous electrolyte and free lauryl alcohol as determined by surface tension studies. It had a melting point of 175–176° (14). A portion of the sample was converted into the free acid by passage through an acid-activated cation exchange resin (Dowex 50) until the filtrate reached a pH of 7. The neutralization equivalent was found to be 292.7; theoretical, 288.4.

Cetyl Trimethyl Ammonium Bromide.—A commercial sample (J. T. Baker Chem. Co.) was twice recrystallized from hot water, m. p. 237–238° (decompn.).

Cetyl Alcohol Polyoxyethylene Ethers.—Commercial samples of the compounds of ethylene oxide content ranging from 10 to 60 moles of ethylene oxide per mole of cetyl alcohol were selected for

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the study.[1] For convenience, the compounds will be designated as C-10, C-14, C-30, and C-60 to denote the average number of moles of ethylene oxide per molecule. All samples were dried at 60° under high vacuum. The C-14 sample was found to contain an impurity which absorbed in the ultraviolet region. This was reduced substantially by fractional crystallization of the compound from absolute diethyl ether, followed by complete removal of any ether held by the compound at 60° with high vacuum. This treatment unfortunately changed the average number of moles of ethylene oxide per molecule of surfactant, since the molecules with the longer polyoxyethylene chain preferentially precipitate.

Apparatus. Beckman DU spectrophotometer with a constant temperature jacketed-cell chamber held at 30° ± 0.1°, and a model 11 Cary ultraviolet recording spectrophotometer.

Analytical Method.—The method employed in the present study consisted essentially of making a series of ultraviolet absorption measurements on a benzocaine solution undergoing hydrolysis at constant temperature and hydroxyl ion conditions. Although the ultraviolet absorption spectrum of benzocaine and its chromophore-containing hydrolytic product, *p*-aminobenzoic acid, are practically identical in neutral conditions, the salt form of *p*-aminobenzoic acid has a different electronic configuration so that its absorption spectrum is sufficiently different to permit quantitative analysis of mixtures of the salt and ester. The per cent of the residual ester was determined by analysis at the point of maximum absorbance of the benzocaine moiety (which shifted in each surfactant system) and at the isosbestic point between benzocaine and *p*-aminobenzoate in 0.04 *N* sodium hydroxide (see Fig. 1). The maxima of the solubilized benzocaine varied from 285 to 296 mμ. The isosbestic point varied from 271 to 277 mμ. The latter were time independent for each system studied as long as secondary oxidative reactions were absent. The solutions were read directly in the silica cuvet without dilutions.

Experimental Procedure.—Stock solutions of the drug and the surfactants were used to prepare 1, 10, and 33 mg. % solutions of benzocaine in the surfactant solutions. The surfactants were studied in concentrations ranging from 0.067 to 10%. The concentration of base was held at 0.04 *N*. Prior to the hydrolysis study, solutions containing the drug and surfactant in the absence of the base were examined for their ultraviolet absorption characteristics, using the Cary model 11 recording spectrophotometer. An identical sample completely hydrolyzed to *p*-aminobenzoate was also studied simultaneously. In this manner the isosbestic point and the absorption maximum were determined explicitly for each system studied.

Each hydrolytic experiment was carried out in the following manner. A volume of stock solution containing an amount of benzocaine equivalent to 1 mg. per 100 ml. of final solution was mixed with an amount of surfactant solution to yield the desired final concentration. These solutions were diluted with water to 14.4 ml. and equilibrated to a temperature of 30° in a constant temperature bath. A 0.6-ml. quantity of 1.000 *N* sodium hydroxide solution was added, and after adequate mixing the solution was placed in the silica cuvet. The latter was transferred into the cell compartment held at a constant temperature by the thermospacer assembly. Nitrogen gas, passed through water at 30°, was circulated in the cell compartment to eliminate carbon dioxide absorption or oxidative changes during the experiment. The solvent cell contained a solution of the surfactant at an identical concentration, to eliminate any possible ultraviolet absorption error due to absorbance of the surfactant. The change of absorbance of the solution with time was followed at the isosbestic point and at the absorption maximum for the benzocaine, as determined on the Cary recording spectrophotometer. Application of the Beer-Lambert relation for binary mixtures was used to calculate the degree of hydrolysis. Known mixtures of benzocaine and its hydrolytic product were prepared and were found to conform to the Beer-Lambert law in the concentrations studied and in the presence of the surfactants. In those experiments where higher concentrations were utilized, silica spacers were used to reduce the path length so that the absorbance was reduced to the sensitivity range of the instrument. In all cases a minimum of a dozen readings was taken for each system. The half lives listed below were obtained from analysis of all the experimental data. Figures 3–5 include only a portion of the data in each case. The error in half-life estimation is approximately ±5% or less.

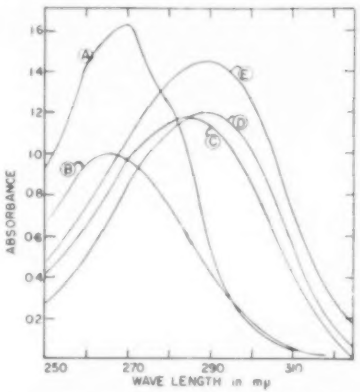

Fig. 1.—Ultraviolet absorption curves: *A*, benzocaine in hexane; *B*, *p*-aminobenzoate ion in 0.04 *N* NaOH; *C*, benzocaine in water; *D*, benzocaine in 0.53% surfactant C-14; *E*, benzocaine in 20% polyethylene glycol 600-water mixture.

RESULTS AND DISCUSSION

The conditions for the hydrolysis of benzocaine selected for this study were the same as were used by Higuchi and Lachman (13) in their study of the hydrolysis of benzocaine in the presence of caffeine, namely, 0.04 *N* hydroxyl ion and 30.0°. The concentration of benzocaine was 10^{-4} *M* or less. While the overall reaction between hydroxyl ion and benzocaine is second order, the experimental

[1] The author wishes to acknowledge the generosity of Mr. C. D. Moore of Glover Chemicals, Ltd., of Leeds, England, for furnishing the samples of surfactants.

conditions lead to an insignificant change on the hydroxyl ion concentration. The observed reaction is, therefore, pseudo first order.

Absorption Spectral Studies of Benzocaine in Various Surfactants.—Figure 1 represents the ultraviolet absorption curves of *p*-aminobenzoate ion in 0.04 *N* NaOH (*B*) and of benzocaine in hexane (*A*), water (*C*), 0.53% nonionic surfactant C-14 (*D*), and in a 20% polyethylene glycol 600-water mixture (*E*). Riegelman and co-workers (15) have previously pointed out that, for certain compounds, the ultraviolet absorption spectrum of a solubilizate affords a sensitive method for determining the four different modes of solubilization which have been mentioned earlier. Changes in spectrum associated with changes in the solvent may be due to (*a*) the influence of polarizability of the valence electrons with changes in the dielectric constant of the environment, (*b*) the influence of the environment on permanent or induced dipoles, (*c*) hydrogen bonding, dimerization, and similar molecular interactions, and (*d*) changes associated with variation in pH. The first two effects are associated with small changes in spectrum, whereas the last two may lead to relatively large changes in spectrum.

Relative to the single absorption band of benzocaine in water, curve *C*, the spectrum of benzocaine in hexane (*A*) illustrates the effect of changes in the polarizability of the valence electron in the molecule. The spectrum is shifted to lower wave length, possesses a larger extinction coefficient, and slightly more fine structure is noted. There appears to be three peaks, at 265 (shoulder), at 270, and at 280 (shoulder). When dissolved in surfactants or 20% polyethylene glycol 600-water mixture, the single absorption band is retained with a slight increase in the extinction coefficient, and the absorption maxima is shifted to the higher wave lengths to the same degree in each solution. From this analysis, it appears that benzocaine, when dissolved in the surfactant, is oriented in the polyoxyethylene exterior of the micelle. One may postulate somewhat on the characteristics of the polyoxyethylene exterior of a nonionic surfactant micelle. It is a well-known phenomenon that these agents in high concentrations form viscous gels wherein the water-swollen micelles are conceived as making direct contact (16). Rösch (17–20) has studied several homologous series of surfactants and found that there are approximately 2 to 2½ moles of water per oxygen atom in the surfactant. Sasaki and co-workers (21) studied the shifts in the absorption bands of selected solubilizates. For the shifts in the *n*, π, and *B* absorption bands, it was concluded that surfactant micelles contain some water molecules in their exterior. Figure 2 lends further evidence to this conclusion. On the lower curve, the absorption maxima for benzocaine solutions is plotted against the log of the concentration of the added surfactant, C-14, and directly against the concentration of polyethylene glycol 600. The concentration of benzocaine was held at 1 mg. % or 10 mg. % during the study. As the concentration of the surfactant is increased, there appears to be a change in the dielectric environment surrounding the drug similar to that which takes place when benzocaine is dissolved in various concentrations of polyethylene glycol 600. Examination of the widths of the absorption curves at half density appear to indicate a negligible contribution from the fraction of the benzocaine which may remain in the water phase, even at the lowest concentration studied. The shift in absorption maximum with concentration of surfactants C-30 and C-60 can be superimposed on the polyethylene glycol curve. As the concentration of surfactant is increased, the exterior of the micelle appears to undergo gradual reorientation to a closer alignment of the individual chains and, in effect, the exterior appears to take on the dielectric characteristic similar to increasing concentrations of polyethylene glycol in water.

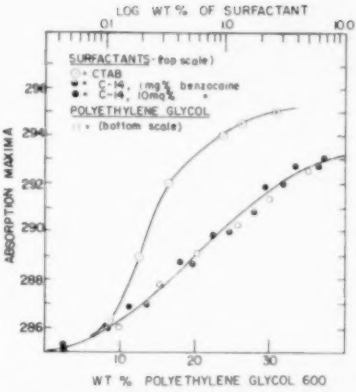

Fig. 2.—A plot showing the shift in the ultraviolet absorption maxima of benzocaine with increasing concentration of surfactant C-14 and in polyethylene glycol 600-water mixtures. The concentration of surfactants is given in log units at the top of the graph. The polyethylene glycol 600 concentration is given in linear units at the bottom of the graph.

The upper curve in Fig. 2 presents the absorption maxima for benzocaine in increasing concentrations of cetyl trimethyl ammonium bromide. It appears that there is an abrupt change in the dielectric environment surrounding the benzocaine as the micelle is formed. This would be expected if the drug is oriented near the exterior of the micelle where coulombic charge of the quaternized nitrogen group may be affecting the valence electrons of the benzocaine moiety. Similar shifts in the absorption maxima were found for sodium lauryl sulfate when the drug is in the presence of the base. The absorption curve of benzocaine in sodium lauryl sulfate solution in the absence of added base is profoundly affected by the environment and a detailed analysis of these changes and their significance will be published later.

Hydrolysis of Benzocaine in Nonionic Surfactant Systems.—Figure 3 and Table I present the results of the hydrolytic studies using surfactant C-14. As the concentration of the surfactant is increased, there is a gradual increase in the half life of the benzocaine under the conditions used in the study. The total concentration of benzocaine which can be incorporated into these systems is relatively large. For example, the water solubility of benzocaine is approximately 40 mg. %, while the 3.3% solution of surfactant C-14 can dissolve as much as 500 mg. %. The two upper curves of Fig. 3 represent data on 3.3% surfactant. The curve with open circles

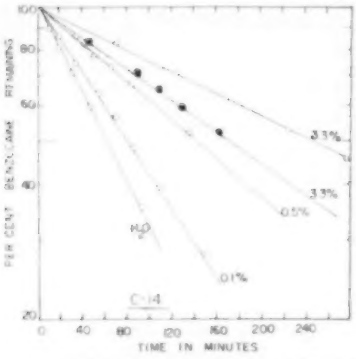

Fig. 3.—A plot showing the effect of increasing concentration of surfactant C-14 on the rate of hydrolysis of benzocaine at 30° and at 0.04 *N* hydroxyl concentration. The curve with closed circles represents the data at 33 mg. % benzocaine; all others at 1 mg. % benzocaine.

TABLE I.—INFLUENCE OF HOMOLOGOUS SERIES OF ALKALI-STABLE NONIONIC SURFACTANTS ON THE HALF LIFE OF BENZOCAINE IN 0.04 *N* NaOH SOLUTIONS AT 30°

Surfactant C-10, %	Half Life, min.	Surfactant C-14, %	Half Life, min.
0.0000	64	0.000	64
0.067	64	0.098	83
.....	...	0.53	145
.....	...	0.80	155
.....	...	3.3	248
.....	...	3.3[a]	177
Surfactant C-30, %	**Half Life, min.**	**Surfactant C-60, %**	**Half Life, min.**
0.000	64	0.000	64
0.067	64	0.067	85
1.33	153	1.33	188
3.3	239	3.3	324

[a] Concentration of benzocaine in this solution was 33 mg. %; all others at 1 mg. %.

represents the data determined when only 1 mg. % of benzocaine was present in the system and the curve with closed circles includes 33 mg. % of benzocaine. Both solutions resulted in pseudo first-order hydrolysis, but the thirty-three fold increase in drug concentration had a relatively small effect on the drug hydrolysis rate. When $^1/_{100}$ of the total saturation concentration was present, the half life was 248 minutes, while when the concentration was $^{33}/_{100}$ of saturation, the half life was 177 minutes. The change in drug-surfactant ratio undoubtedly influences the amount of the drug in the free state in the water environment. If a significant portion of the benzocaine were in the water phase, the absorption curve should indicate an inflection at wave lengths below the maximum relative to the placement of curves *C* and *D* in Fig. 1. It should also be detectible by comparison of the widths of the absorption curves at half density. Study of the absorption curves indicates that the drug is almost completely in the micelle, yet the half life is increased less than fourfold relative to the surfactant-free system even though the solution is $^1/_{100}$ of saturation. In accordance with the relatively high degree of hydration of the surface of the micelle, it would appear that hydrolytic attack is taking place within the micelle as well as in the aqueous phase.

Table I includes the data on the influence of the nonionic surfactant from C-10 to C-60 and on the half life of benzocaine in 0.04 *N* NaOH solution at 30.0°. The C-10 surfactant is poorly soluble in water and cannot be used in the spectrophotometric analysis procedures at higher concentrations. The apparent discrepancy in the half life with concentration of the C-14 surfactant relative to surfactant C-30 is probably due to the fact that the surfactant designated as C-14 was raised in its polyoxyethylene content by the fractional crystallization procedure required to remove the ultraviolet absorbing impurities.

There is a relatively small change in the rate of hydrolysis as one changes to a surfactant with longer polyoxyethylene chain (see Table I). It is well known that the critical micelle concentration increases with chain length, yet the hydrolysis rate appears to be affected by concentration changes to a greater extent than by changes in the hydrophilic-hydrophobic balance of the nonionic surfactant. However, different ratios of the drug to surfactant may change this conclusion.

Hydrolysis Studies of Benzocaine in Charged Micellular Systems.—The influence of increasing concentration of sodium lauryl sulfate on the hydrolysis rate of benzocaine is shown in Fig. 4 and in Table II. The solution containing 0.267% of sodium lauryl sulfate is approximately at the critical micelle concentration for the surfactant, 0.25% (14). The effect of the association of benzocaine close to the anionic head group of the surfactant is made a definite barrier to the approach of the hydroxyl group. The drug has been stabilized such that the half life is increased to 104 minutes. As the concentration is further increased, however, the effect is even more pronounced and the half life is extended to 1,150 minutes when the concentration is increased to 5% sodium lauryl sulfate. Under these conditions, the polar head group apparently plays a predominant role in hindering penetration of the hydroxyl group into the micelle.

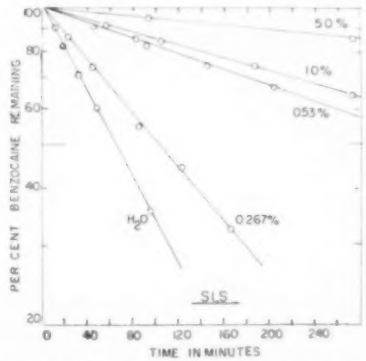

Fig. 4.—A plot showing the effect of increasing concentration of sodium lauryl sulfate on the rate of hydrolysis of benzocaine at 30° and at 0.04*N* hydroxyl ion concentration. All curves at 1 mg. % benzocaine.

TABLE II.—INFLUENCE OF ANIONIC AND CATIONIC SURFACTANTS ON THE HALF LIFE OF BENZOCAINE[a] IN 0.04 *N* NaOH SOLUTIONS AT 30°

Sodium Lauryl Sulfate, % Concentration	Half Life, min.
0.000	64
0.267	100
0.533	350
1.00	420
5.00	1,150
Cetyl Trimethyl Ammonium Bromide, % Concentration	**Half Life, min.**
0.000	64
0.067	57
1.34	425
2.46	650

[a] One mg. % concentration in all cases.

Figure 5 and Table II represents similar data of increasing concentrations of cetyl trimethyl ammonium bromide. At higher concentrations, the benzocaine appears to be shielded by the cationic polar head group. Although the negatively charged hydroxyl ion is attracted by the cationic group, it apparently cannot penetrate beyond this polar head into the deeper confines of the micelle wherein the benzocaine appears to be held. With both ionic surfactants, the predominant side for hydrolysis may be the free aqueous environment surrounding the micelles. Further work is necessary to confirm this latter postulate, however.

It should be particularly noted, however, that the lowest concentration of the quaternary studied, 0.067%, appeared to increase the hydrolysis rate rather than produce stabilization as was noted in other cases. This concentration is slightly above the CMC of 0.033% (22, 23). In a loose arrangement of chains which must be found in the initial stages of micelle formation, it is possible for the cationic head group to attract the hydroxyl ion to the environment of the benzocaine and thereby to produce an increase in the hydrolysis rate.

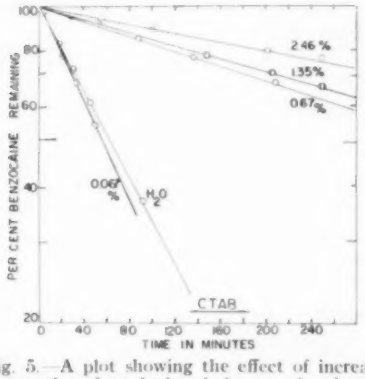

Fig. 5.—A plot showing the effect of increasing concentration of cetyl trimethyl ammonium bromide on the hydrolysis of benzocaine at 30° and at 0.04 *N* hydroxyl ion concentration.

SUMMARY

1. The surface of a micelle of nonionic surfactants appears to be relatively highly hydrated, equivalent to 5–35 per cent polyethylene glycol-water mixtures.

2. Benzocaine is solubilized in the palisade layers within the region of the hydrated polyoxyethylene chains.

3. At the drug-surfactant ratios studied, the rate of hydrolysis of benzocaine is more dependent on the concentration of the nonionic surfactant than on the length of the polyoxyethylene chain within this homologous series.

4. Hydroxyl ions apparently penetrate into the polyoxyethylene portion of the micelle, since the hydrolysis appears to be taking place in this environment as well as in the water layer.

5. In micelles composed of cationic or anionic surfactants, the polar head group apparently shields the benzocaine from direct base catalysis. These agents produce the most profound change in the hydrolysis rate extending the half life eighteen-fold in a 5 per cent solution of sodium lauryl sulfate.

6. Low concentrations of the quaternary, cetyl trimethyl ammonium bromide, just above the CMC cause a slight acceleration in the rate of hydrolysis, probably attracting the hydroxyl ion to the benzocaine environment.

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A New Method of Manufacture of Tablet Granulations I*

By P. A. TUERCK, E. L. WALTERS, and E. D. CARKHUFF

A method of manufacture of tablet granulations has been developed in our laboratories which reduces granulating operations, manufacturing time, and material costs. Granulations produced by this new method have been divided into two broad classifications: low milligram potency ingredients, where a major portion of the tablets may be diluent, and high milligram potency ingredients, where active ingredients constitute a major portion of the tablet. This paper is concerned with a discussion of the low milligram potency formulations. The method involves the use of standard coating pans in which granulations are prepared by spraying with water and subsequent drying in the pan.

WHETHER THE STANDARD METHOD of manufacture be wet granulation or dry slugging with recompression, considerable time, labor, and space are involved in preparing granulations for the compressing machines. Experimental, pilot plant, and production work have been performed on a method which presents many advantages over conventional methods now employed.

In the study presented here we have attempted to describe the method and point out some of its advantages.

EXPERIMENTAL

In the manufacture of a tablet containing a low milligram potency ingredient, the active ingredient is reduced to a fine powder by micropulverizing. This powder is introduced into a coating pan equipped with baffles and a variable speed drive. A diluent, such as powdered sugar containing 3% starch, is added. Powders are mixed by tumbling at a relatively high speed. While tumbling, the powders are sprayed with an amount of water sufficient to cause formation of small granules. Using powdered sugar as an example, this amount is equivalent to 3 4% of the total weight of the granulation. The spray must be in the form of a fine mist. As the powders are wetted, the speed of the pan is gradually reduced to prevent impaction at the periphery due to centrifugal force. Tumbling of the powders results in formation of small round granules. The speed of the pan is reduced to about 3 r. p. m. and a gentle current of hot air is blown over the tumbling granulation. In the case of powdered sugar, it is necessary to remove only about 2% moisture to obtain a granulation with satisfactory tabletting characteristics. A suitable lubricant, such as magnesium stearate, is then added, completing the operation. In practice we have noticed a small percentage of the granulations to be over 10 mesh in size. A major portion of the granulations is less than 30 mesh in size. To obviate any large lumps, the finished granulation is sifted through an oscillating grinder equipped with a 10-mesh screen.

We have tested this method on a laboratory, pilot plant, and a production scale. It is possible to manufacture and run a granulation on a compressing machine well within one hour, on an experimental or pilot plant scale, involving batches of 0.1 to 5 Kg. in size. It is possible to duplicate this operation on production batches of 200 Kg. within a period of four hours.

APPARATUS

Experimental Equipment. In preparing very small batches of about 200 Gm., a stainless steel coating pan 6.5 inches in diameter was used. The pan was mounted on a shaft driven at a constant speed of 30 r. p. m. Water was sprayed on by use of a DeVilbiss atomizer No. 127, of the type ordinarily used for nasal and throat sprays. The bulb was removed and a hose was connected to an air pressure line to provide a steady and continuous flow for uniform atomization. The expelled air tended to blow particles from the pan unless the pressure was carefully regulated to a very low pressure. After granules were formed, the granulation was dried to a moisture content suitable for satisfactory compression with a hot air gun. Due to the excessive velocity of the air output of the heat gun, sufficient drying was obtained by directing the hot air to the outside of the pan, thereby preventing the granulation from being blown out. We feel there is a great possibility that heat applied to the outside of a production size pan would be all that is needed to dry large batches of granulations with a current of air provided. This will be investigated. A hand screen of convenient size is used to sift the finished granulation. The mesh size may be 10 to 14, depending on the size of the contemplated tablet, the smaller mesh size being used for smaller tablets.

Pilot Plant Equipment. A Raymond Mill was used for micropulverizing the ingredients, although any other micropulverizing apparatus would be suitable. Stainless steel or copper coating pans, 18 inches in diameter, equipped with baffles, were used. The pans were mounted, interchangeably, on a shaft attached to a d.-c. gear head motor. Speed of the pan was controlled from 0 to 30 r. p. m. by means of a Variac speed control (General Radio Co., Cambridge, Mass.). The hot air gun previ-

* Received August 21, 1959, from the Research Laboratories of the Wm. S. Merrell Company, Cincinnati, Ohio.
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The authors wish to thank Mr. Truman Sheldt for his able technical assistance in carrying out this project.

ously described was used for the drying operation. Temperature and rate of flow were controlled by a rheostat. Hand screens of suitable mesh sizes were used in the final sifting operation.

Production Equipment.—Ingredients were first reduced to a fine powder by passing them through a Fitzpatrick comminuter. A 48-inch baffled copper pan was provided with a variable speed drive in the range of 3 to 30 r. p. m. to accommodate production size batches of approximately 135 Kg. Batch size may be substantially increased by using larger pans. Naturally, stainless steel pans would provide obvious advantages over copper with respect to inertness, sanitation, etc. A hot air dryer was fabricated to deliver air at a temperature of 375°F. and a rate of 226 c. f. m. The temperature and rate of flow were variable. Initially, water was sprayed on the granulations by means of pneumatic guns. While such guns provide fine atomization, air pressure must be carefully regulated to prevent blowing granulations from the pans. This difficulty may be circumvented by using suitable spray nozzles which may attach directly to a water line by means of a hose and which provide satisfactory atomization with pressures of 40 to 80 psig. The dried, lubricated granulations are sifted and ground directly into storage cans through oscillating grinders equipped with suitable screens, or the finished granulations may be transferred directly to a tablet machine.

METHOD

Formulations.—As a means of illustrating advantages of this new method of manufacture, a series was prepared utilizing some common diluents to prepare placebo granulations by the described method as well as by a conventional method in a Day mixer.

A series of eight experimental granulations was prepared by spraying the powders described below with water. A water-soluble dye, D&C Green No. 5, was first mixed with each powder in a concentration of 0.5 Gm. per kilogram and micropulverized in a Raymond mill before granulating. This color was chosen because it usually causes difficulty in producing smoothly colored tablets when granulated by ordinary wet granulating methods. In all cases batch sizes of 2.5 Kg. were used as a matter of convenience for processing in existing pilot plant equipment. The wet granulations thus formed were dried while tumbling in the coating pan, lubricated with 1% magnesium stearate, and compressed on a model RB-2 Stokes single rotary tabletting machine using $^{13}/_{32}$ inch standard cup punches and dies at a tablet weight of 400 mg.

In like manner the series was repeated, granulations being made in a small Day mixer by adding water as a granulating agent. Wet granulations were forced through a 6-mesh screen and dried overnight on trays in a hot air oven at 120°F. Dried granulations were ground through a 12-mesh screen, lubricated, and compressed as previously described.

Materials comprising the series are as follows: (*A*) powdered sugar; (*B*) powdered sugar 90%, corn starch 10%; (*C*) lactose; (*D*) lactose 90%, corn starch 10%; (*E*) mannitol 98%, powdered gelatin 2%; (*F*) mannitol 88%, powdered gelatin 2%, corn starch 10%; (*G*) powdered sugar 50%, lactose 25%, corn starch 25%; (*H*) powdered sugar 90%, corn syrup solids 10%.

Table I compares granulations and tablets obtained by both methods with respect to moisture content, drying times, mesh sizes, compression characteristics, hardness, disintegration, and general appearance. Advantages of the spray granulation method over the conventional method described are evident in this table. It is interesting to note that in spite of a high percentage of "fines" in some instances (spray granulations *C*, *D*, *E*, *F*), excellent tabletting characteristics were maintained.

In the interests of simplicity, stability, and economy, a plain powdered-sugar base is satisfactory for the majority of low milligram potency medicaments.

Examples of formulations successfully prepared with a sugar base are phenobarbital tablets (15 mg., 30 mg., 60 mg., 90 mg.), thiamine hydrochloride tablets (10 mg., 25 mg., 50 mg., 100 mg.), ascorbic acid tablets (50 mg., 100 mg.), and a variety of specialty products.

Coloring.—Granulations may be colored simply by micropulverizing pigments, lakes, or dyes along with the other tablet ingredients as they are being introduced in the pan. Although water-soluble dyes may be distributed on a portion of the diluent and dried prior to grinding, we have found that even this step may be eliminated by using dyes soluble in volatile solvents, such as alcohol or chloroform, and merely pouring them over portions of the sugar prior to micropulverizing. Naturally, the micropulverizing equipment must be hooded and ventilated to eliminate explosion or health hazards. Any residual solvent is driven off during the drying operation in the coating-pan.

Particle Size.—The mesh size of the final granulation may be controlled by regulating the amount of moisture sprayed on the granulation and by the length of time the moist granulation is tumbled in the coating pan.

If the granulation is overwet or tumbled too long, large balls result. If underwet, the granulation may have a very large proportion of "fines."

When properly made, granulations prepared by this method lend themselves admirably to high speed tabletting operations. Characteristics of the granulations are such that embossing or bisecting present no problems.

Lubrication.—A variety of lubricants have been used. Magnesium stearate was satisfactory, although other stearates and talc have been used. After introduction of the lubricant to the batch, a final spraying of 0.25 to 0.5% of light mineral oil has been found to eliminate further dusting and to provide a gloss to the tablet.

Processing Time.—As previously mentioned, a 135-Kg. batch of granulation may be completed to the point of compressing within a three or four-hour period. Spraying time may be accomplished within ten minutes; drying time within one or two hours. The major portion of manufacturing time is required for the manual charging of the pan and removal of the finished granulation from the pan. Refinements are possible to shorten these times further. By increasing the temperature of the air delivered by the drying gun, the drying

TABLE I.—COMPARISON OF GRANULATIONS AND TABLETS MADE BY THE SPRAY GRANULATING METHOD WITH THOSE PREPARED IN A DAY MIXER

		Granulations				Classification of Screen Mesh Sizes of Dry, Ground Granulations, %							Tablets			
	Method	Granulating Solution Used (Water), ml	% Moisture in Wet Granulation (Karl Fischer)	Drying Times, hr.	% Moisture in Dry Granulation (Karl Fischer)	20	30	40	50	100	200	<200	Compression Characteristics[a]	Hardness (Monsanto)	Disintegration U. S. P. Method, Min. 37° Water	General Appearance[b]
A	Spray	120	3.06	0.25	0.42	22	13	15	13	17	12	8	1	9	5.5	1
A	Day mixer	185	6.9	18	0.53	52	13	9	8	10	6	2	2	8	5	4
B	Spray	125	5.17	0.17	1.02	14	8	10	12	20	17	19	1	7.5	3	1
B	Day mixer	200	8.7	18	1.02	58	12	8	6	11	4	1	2	7	9.5	4
C	Spray	250	11.48	0.17	5.47	20	5	3	3	3	10	56	1	9.5	10	1
C	Day mixer	365	18.68	18	4.71	47	14	9	7	10	5	8	3	5	15	4
D	Spray	275	11.31	0.17	6.14	9	3	3	2	5	15	63	1	9	1.5	1
D	Day mixer	535	22.04	18	4.66	59	15	9	6	7	3	1	2	7	1	4
E	Spray	250	6.53	0.5	0.48	41	9	5	5	9	7	24	1	12.5	9	2
E	Day mixer	675	20.6	18	0.48	46	14	10	8	11	5	6	2	11.5	9	4
F	Spray	275	7.11	0.5	1.33	12	4	3	2	10	14	55	1	9.5	0.75	1
F	Day mixer	800	25.83	18	0.51	60	12	6	4	9	8	1	2	9.5	6	4
G	Spray	215	9.36	0.5	2.02	12	5	7	8	23	28	17	1	9	3	2
G	Day mixer	260	11.7	18	5.19	26	10	10	12	29	12	1	2	7	3	4
H	Spray	100	3.38	0.25	0.53	5	4	7	12	46	22	4	1	9.5	5.5	3
H	Day mixer	165	6.73	18	1.12	63	15	8	5	5	3	1	2	7.5	7.5	4

[a] 1, good; 2, fair; 3, poor (capping).
[b] 1, Excellent; 2, good; 3, fair (slightly mottled); 4, poor (badly mottled and spotted).

TABLE II.—A COMPARISON OF MANUFACTURING TIMES REQUIRED FOR PRODUCTION SIZE BATCHES OF TABLET GRANULATIONS MADE IN A DAY MIXER AND BY THE SPRAY TECHNIQUE

	Phenobarbital, 15 mg.				Thiamine HCl, 10 mg.				Ascorbic Acid, 50 mg.			
	Day Mixer		Spray Method		Day Mixer		Spray Method		Day Mixer		Spray Method	
	Labor Time, hr.	Total Mfg. Time, hr.	Labor Time, hr.	Total Mfg. Time, hr.	Labor Time, hr.	Total Mfg. Time, hr.	Labor Time, hr.	Total Mfg. Time, hr.	Labor Time, hr.	Total Mfg. Time, hr.	Labor Time, hr.	Total Mfg. Time, hr.
Loading and granulating	1.5	1.5	0.5	0.5	0.75	0.75	0.25	0.25	1.0	1.0	0.4	0.4
Wet screening	1.75	1.75	..	..	1.0	1.0	..	..	1.0	1.0	..	..
Drying		16		2		16		1.25		16		1.5
Dry screening	1.0	1.0	0.5	0.5	1.0	1.0	0.25	0.25	0.25	0.25	0.3	0.3
Lubrication	0.75	0.75	0.1	0.1	0.5	0.5	0.1	0.1	0.25	0.25	0.1	0.1
Total cleanup	0.75	0.75	0.25	0.25	1.0	1.0	0.25	0.25	0.5	0.5	0.25	0.25
Total	5.75	21.75	1.35	3.35	4.25	20.25	0.85	2.1	3	19	1.05	2.55
Labor time saved			4.4	..			3.4	..			1.95	..
Production time saved				18.4			..	18.15			..	16.45
Batch size		2,750,000				300,000				400,000		
Total wt. of material		214 Kg.				89.5 Kg.				112.5 Kg.		

cycle may be further shortened. When a high level of starch is used, more water is needed for granulation and the drying time is slightly longer. However, with regard to compression characteristics, the amount of moisture is not as critical as in a straight sugar granulation.

As a means of illustration, Table II emphasizes savings effected in production time on three typical U. S. P. tablet granulations. Comparisons are drawn between batches made on a production scale by a conventional wet granulating method in a Day mixer and batches made in a coating pan using the spray technique. It must be realized that methods of granulation and production techniques will vary considerably from company to company. Data contained in Table II are, therefore, relative only to our particular production methods.

Stability.—A considerable number of tablet granulations are currently manufactured which, because of heat and moisture lability, require protracted drying times in dehumidified areas to preserve stability. Many such granulations may be safely made by our method, since a smaller amount of moisture is introduced into the granulation, and since it is dried to a satisfactory level for compressing within a short period of time. Although high temperatures (160–500°F.) may be used in the drying operation, our experience has been that the temperature within the granulation is maintained at safe limits, because of the rate of evaporation of the granulating agent, until the granulations begin to dry. Naturally, a close check must be kept on moisture content to prevent overdrying with a resultant increase in temperature. Furthermore, if an anhydrous base is required, tablets may be packaged immediately with silica gel packets for further moisture reduction.

Tabletting.—Granulations prepared as described may be run at high speeds. Due to the soft nature of the granulations, wear on punches, dies, and other machinery is reduced to a minimum.

Disintegration.—Tablets prepared by the outlined

method dissolve rather than disintegrate. The solution or disintegration time has not been found to exceed fifteen minutes when tested by the U. S. P. XV method.

CONCLUSIONS

1. A new method for manufacturing granulations of low milligram potency medicaments has been developed.

2. The method involves the use of standard coating pans in which granulations are formed by spraying with water and subsequent drying in the pan.

3. The method has advantages of decreasing material costs, reducing manufacturing time, and eliminating a number of processing steps, resulting in economies in labor and space.

A New Method of Manufacture of Tablet Granulations II*

By P. A. TUERCK, E. L. WALTERS, and E. D. CARKHUFF

This paper is concerned with a discussion of a method of manufacture for tablet granulations in which active ingredients comprise a major portion of the tablet. The method involves the use of standard coating pans in which granulations are formed by spraying with starch paste or other commonly used granulating solutions and subsequent drying in the pan.

IN SOME INSTANCES, due to limitations in space and because rapid disintegration is required, a modification of the granulating method described in Part I is desired.

The method described in Part I principally utilizes sugar and other water-soluble ingredients as diluents. Tablets resulting from such granulations dissolve rather than disintegrate.

We have pointed out in the description of the method of manufacture that granulations containing major proportions of sugar and other water-soluble ingredients may be made by spraying with water and drying while the coating pan is continuously rotated. This is possible because of the durability of the granulation formed.

We have found that in instances where active ingredients form a major portion of the tablet and where rapid disintegration is desired (A. P. C. tablets, for example), it is desirable to spray granulate with starch paste. In other formulations, gelatin, glucose, sorbo, and other granulating agents may be preferred.

In some instances, particularly when starch paste is the granulating agent, we have noticed that, when the procedure outlined in Part I was followed, a satisfactory granulation could not be obtained. This result may be attributed to the fact that starch granulations are extremely soft until dry. The continuous tumbling action in the coating pan reduced the wet granulation to a fine powder. A further complicating factor was the necessary use of a considerably larger amount of granulating agent in this type of formulation which increased drying time and, hence, aggravated the formation of "fines."

An interesting modification of the procedure is also being investigated wherein dry soluble starch or other dry gums are mixed with the powdered ingredients before granulating. By spraying the granulation with water, starch paste or gum solutions are produced *in situ*. This expedient permits granulation with lesser amounts of moisture and is particularly effective in maintaining smoothness of color when a colored tablet is desired.

In like manner, dry ethyl cellulose may be mixed with ingredients and the granulating solution produced *in situ* by spraying with ethyl or isopropyl alcohol.

APPARATUS

To circumvent the difficulty, two changes were made in the equipment. First, the hot air source was modified so that the temperature of delivered air was 400–600°F. This experiment shortened the drying time by increasing the rate of evaporation. Secondly, a timing mechanism was placed on the motor

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of the pan which automatically rotated the pan one revolution every minute. This was put in operation only during the drying cycle. Depending upon the drying characteristics of different granulations, the intervals at which the pan is rotated may be easily changed by adjusting the timing mechanism.

This expedient allowed the exposed surface to dry during each resting cycle. The time the granulation was in motion was greatly reduced, thereby eliminating formation of too many "fines."

With these exceptions, equipment and procedure is the same as outlined in Part I.

EXPERIMENTAL

Granulations (A, B, and C) of high milligram potency materials were prepared by both the spray granulation technique and by a conventional method of wet granulating in a Day mixer as illustrated below. The granulations as well as tablets prepared from them are compared in Table I.

GRANULATION A—A. P. C.

	Day Mixer Method, Kg.	Spray Method, Kg.
Acetophenetidin	2.52	2.52
Caffeine	0.5	0.5
Corn starch	0.72	0
Soluble starch (Corn Products Refining Co.)	0	0.72
Add at time of lubrication:		
Aspirin granulation (16% starch)	4.6	4.6
Corn starch *q. s.* to	8.7	8.7

Day Mixer Method.—Mix and granulate ingredients with a solution composed of 10% starch paste and 16% gelatin. Wet screen through a 6-mesh screen. Spread on trays and dry overnight at 120°F. Dry screen through a 12-mesh screen. Add aspirin granulation and corn starch lubricant. Compress on $^{7}/_{16}$ inch standard cup punches at a weight of 518 mg.

Spray Method.—Mix ingredients in a coating pan and granulate by spraying with water. Dry, add aspirin granulation, and lubricant to pan. Sift through 10-mesh screen. Compress as above.

GRANULATION B—SULFADIAZINE, 500 MG. PER TABLET

	Day Mixer Method, Kg.	Spray Method, Kg.
Sulfadiazine	3	3
Corn starch	0.34	0.3
Corn syrup solids	0	0.075
Soluble starch (Corn Products Refining Co.)	0	0.15

Day Mixer Method.—Mix and granulate with a solution composed of 5% starch paste and 5% glucose. Wet screen through a 6-mesh screen. Dry overnight on trays at 120°F. Dry screen through a 12-mesh screen. Lubricate with 1% magnesium stearate and compress at 616 mg., using $^{7}/_{16}$ inch standard cup punches.

Spray Method.—Mix ingredients in coating pan. Granulate by spraying with water. Dry and lubricate in the pan. Compress as above.

GRANULATION C—ASCORBIC ACID, 250 MG. PER TABLET

	Day Mixer Method, Kg.	Spray Method, Kg.
Ascorbic acid, powdered	1.605	1.605
Sugar, powdered	0.54	0.54
Lactose	0.816	0.816
Acacia, U. S. P. white powder	0.018	0.018
Corn starch	0.093	0
Soluble starch (Corn Products Refining Co.)	0	0.093

Day Mixer Method.—Mix and granulate ingredients with 16% gelatin solution. Wet screen through a 6-mesh screen. Spread on trays and dry overnight at 120°F. Dry screen through a 12-mesh screen. Lubricate with 1% magnesium stearate. Compress on $^{7}/_{16}$ inch standard cup punches at a weight of 544 mg.

Spray Method.—Mix ingredients in a coating pan and granulate by spraying with water. Dry and lubricate in the pan and compress as above.

FORMULATIONS

Although a major portion of our work relating to the high milligram potency type of granulation has been with a starch paste granulating agent, instances have arisen where modifications were necessary.

Some water-insoluble materials, particularly when fluffy or crystalline in nature, require other granulating agents. In some instances granulation may be accomplished by merely spraying with a volatile solvent for the material in question. In other cases it is necessary to add additional binders, such as ethyl cellulose, methyl cellulose, polyvinylpyrrolidone, etc., to the solvent before spraying. Although the cost of the solvents presents a disadvantage, drying time is considerably shortened.

As might be expected, care must be taken in proper selection of disintegrating agents, each granulation presenting its individual problem.

PROCESSING TIME

Since starch paste granulations require substantially more granulating agent for satisfactory results, we have found it necessary to use longer periods of time for the drying operation. Drying may be accomplished overnight by the method described. In practice, we have found that for scheduling purposes it is more convenient to manufacture the sugar base or fast drying granulations during the day and to spray the slower drying starch paste granulations near the end of the day's operations, thereby allowing an overnight period for drying. We are currently investigating refinements which will shorten drying time.

Table II illustrates the difference in manufacturing time required on production size batches when the spray method is compared with wet granulation in a Day mixer. Economies in both labor and total manufacturing time are effected in the spray method

TABLE I.—COMPARISON OF GRANULATIONS AND TABLETS MADE BY THE SPRAY GRANULATING METHOD WITH THOSE PREPARED IN A DAY MIXER

		Granulations												Tablets			
	Method	Granulating Solution Used, ml.	Moisture in Wet Granulation, %	Drying Time, hr.	Moisture in Dry Granulation, %	Classification of Screen mesh Sizes of Dry, Ground Granulations, % 20	30	40	50	100	200	<200	Compression Characteristics[e]	Hardness (Monsanto)	Disintegration, U.S.P. Method, 37°, Water, min.	General Appearance[f]
A	Spray	450[a]	9.4[c]	0.5	2.19[c]	33	12	8	3	16	20	8	1	10	0.25	1
A	Day mixer	1,600[b]	26.4[c]	18	1.91[c]	60	18	9	4	4	3	2	1	8.5	0.7	2
B	Spray	1,200[a]	22.4[c]	0.7	1.8[c]	5	2	3	7	52	27	4	1	13	15	1
B	Day mixer	1,600[b]	22.4[c]	18	1.4[c]	12	10	10	10	22	23	13	1	13	13	1
C	Spray	200[a]	6[d]	0.25	3.8[d]	22	10	9	8	21	17	13	1	12	6	1
C	Day mixer	225[b]	8.5[d]	18	4[d]	60	15	10	6	6	2	1	2	9	8	2

[a] Water.
[b] Refer to formula.
[c] Karl Fischer.
[d] Twenty-four hour drying at 100°.
[e] 1, Good; 2, capping tendency.
[f] 1, Good; 2, spotty, chipping tendency on edges.

TABLE II.—A COMPARISON OF MANUFACTURING TIMES REQUIRED FOR PRODUCTION SIZE BATCHES OF TABLET GRANULATIONS MADE IN A DAY MIXER AND BY THE SPRAY METHOD

	A. P. C.				Sulfadiazine, 500 mg.				Ascorbic Acid, 250 mg.			
	Day Mixer		Spray Method		Day Mixer		Spray Method		Day Mixer		Spray Method	
	Labor Time, hr.	Total Mfg. Time, hr.	Labor Time, hr.	Total Mfg. Time, hr.	Labor Time, hr.	Total Mfg. Time, hr.	Labor Time, hr.	Total Mfg. Time, hr.	Labor Time, hr.	Total Mfg. Time, hr.	Labor Time, hr.	Total Mfg. Time, hr.
Loading and granulating	1.5	1.5	0.5	0.5	1.5	1.5	0.4	0.4	1.0	1.0	0.4	0.4
Wet screening	2.0	2.0	...	...	2.25	2.25	...	...	1.5	1.5	...	...
Drying	...	16	...	2.0	...	16	...	1.5	...	16	...	1.5
Dry screening	1.0	1.0	0.4	0.4	1.5	1.5	0.3	0.3	0.5	0.5	0.3	0.3
Lubrication	1.25	1.25	0.5	0.5	0.25	0.25	0.1	0.1	0.25	0.25	0.1	0.1
Total clean-up	0.75	0.75	0.25	0.25	1.0	1.0	0.25	0.25	0.25	0.25	0.25	0.25
Total	6.5	22.5	1.65	3.65	6.5	22.5	1.05	2.55	3.5	19.5	1.05	2.55
Labor time saved	...	...	4.85	...	...	...	5.45	...	...	...	2.45	...
Production time saved	...	...	...	18.85	...	...	...	19.95	...	...	...	16.95
Batch size		840,000				200,000				200,000		
Total wt. of material, Kg.		435				123				109		

by eliminating certain steps in manufacture, cutting down on clean-up time, and shortening drying time.

Coloring, granulating, lubricating, and tabletting characteristics of the granulations discussed may be considered to be substantially the same as those discussed under Part I.

The wear on punches, dies, and machinery depends to a large extent on the abrasiveness and physical characteristics of the active ingredient. We have found that our method has advantages over standard methods, particularly when a binder is present in the granulating solution. Since, by our method, the granulating agent is sprayed on the ingredients, they tend to be coated with the binding agent, thereby minimizing the abrasiveness or gumminess of the granules. We have prepared granulations which caused binding when standard techniques were used, but which compressed with no difficulty when spray granulating was employed.

DISINTEGRATION AND STABILITY

Tablets made by the method outlined in Part II usually disintegrate rather than dissolve.

Since there is no major change in diluents by this method, stability of tablets is comparable to that of tablets made by standard procedures.

CONCLUSIONS

1. A new method for manufacturing granulations of high milligram potency medicaments has been developed.

2. The method involves the use of standard coating pans in which granulations are formed by spraying with starch paste or other commonly used granulating solutions, with subsequent drying in the pan.

Esterification of Two Sterically Hindered Acids Using Ultrasound Waves*

By ROBERT J. GERRAUGHTY† and PAUL J. JANNKE

The possibility of using ultrasound waves to effect the direct esterification of sterically hindered acids was explored. The methyl, ethyl, *n*-propyl, and *n*-butyl esters of *o*-thymotic and ursolic acids were prepared by the application of low frequency ultrasound waves, the rates of esterification were determined, and the mechanism of the reaction was studied, using methyl mercaptan in place of an alcohol. Branched chain esters of the acids could not be prepared even when exposure conditions were broadly varied.

NUMEROUS STUDIES have been conducted on the direct esterification of sterically hindered acids. The success of the reaction varies over a broad range, depending, in part, upon the structures involved and the conditions applied. Nevertheless, esters of many of these acids have been prepared, frequently by indirect methods. The application of ultrasound waves to overcome steric hindrance was deemed advisable because it has been shown that such energy is capable of stimulating a wide variety of chemical reactions, some of them involving factors associated with esterification.

The kind of energy used is of utmost importance because high frequencies and cavitation favor the hydrolysis of esters (1–4); therefore it appeared logical to assume that low frequencies and lessened cavitation might favor ester formation.

This study employed two acids, one of which is strongly hindered, the other being more moderately so. *o*-Thymotic acid is strongly hindered because of substituents occupying both positions *ortho* to the carboxyl group. Attempted condensations of it with alcohols in the presence of strong dehydrating agents lead to the formation of thymotides (5) instead of the conventional esters. Ursolic acid, which is a pentacyclic alicyclic acid, shows steric hindrance because of the spatial orientation of the cyclic system adjacent to the ring which carries the carboxyl group (6).

A method for the esterification of the two acids is described and the results are shown in Tables I and II. Rates of esterification of both acids were determined. Table III indicates that esterification of ursolic acid is gradual at first, reaches a maximum after seventy-five minutes, and declines rapidly after ninety minutes. *o*-Thymotic acid (Table IV) also is esterified slowly at first, reaching a maximum rate after one hundred and five minutes, and then declining rapidly.

A study of the mechanism of the reaction was conducted to determine if ultrasound altered it in any way. Under normal conditions in direct esterification, the acid acts as a hydroxyl donor and the alcohol as a hydrogen donor (7). This appears not to be the case when esterification is accomplished under the influence of ultrasound. The condensation of the acids with methyl mercaptan proceeded in such a manner as to result in the formation of hydrogen sulfide, indicating clearly that under these conditions the acids served as hydrogen donors, otherwise water would have been formed.

EXPERIMENTAL

Preparation of Ursolic Acid Esters.—Fifty milliliters of the selected alcohol was degassed by exposure[1] at 80 to 85 ma. plate current and 39.1 kc. for one hour. One gram of the ursolic acid, 2 drops of concentrated sulfuric acid catalyst, and 2 Gm. of anhydrous copper sulfate (to remove water formed in the reaction) were added to the degassed alcohol. The polyethylene bottle used as the exposure vessel was immersed below the surface of the water in the transducer tank to decrease cavitation, and the mixture was exposed at not more than 45 ma. plate current for two hours, and then filtered. The solvent was evaporated and the residue was dissolved in ether. The ethereal solution was washed with three successive 100-ml. portions of 3% sodium hydroxide solution and then washed twice with 100-ml. quantities of distilled water. The ether was evaporated and white crystalline ursolic acid ester was recrystallized twice from 70% ethanol. Straight chain esters were prepared by this method in good yields (Table I).

Branched chain esters could not be prepared even by extending exposure periods or increasing the plate current.

Carbon-hydrogen determinations were made according to the method of Niederl and Niederl (8).

Preparation of acetyl derivatives of the esters by refluxing with acetic anhydride for one hour yielded acetyl ursolic acid esters that compared favorably with those reported in the literature, as shown in Table I.

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[1] "Sonogen" Ultrasonic Generator, Model 25-B, Branson Ultrasonic Corp., Stamford, Conn.

TABLE I.—URSOLIC ACID ESTERS

Ester	% Yield[a]	Melting Point, ° C.—Acetyl Esters—Reported (10)	Melting Point, ° C.—Acetyl Esters—Found	Melting Point, ° C.—Acetyl Esters—Reported (11)	Melting Point, ° C.—Acetyl Esters—Found	Carbon and Hydrogen Analysis, %—Calcd—C	Carbon and Hydrogen Analysis, %—Calcd—H	Carbon and Hydrogen Analysis, %—Found[b]—C	Carbon and Hydrogen Analysis, %—Found[b]—H
Methyl	80.1	169–170	170–171	245	243–244	79.15	10.64	79.0	10.7
Ethyl	67.1	...	198–199	194	192–193	79.32	10.74	79.1	10.6
n-Propyl	47.9	...	190–191	173	171–172	79.52	10.84	79.4	10.8
n-Butyl	41.0	...	180–182	123–125	124–126	79.69	10.94	79.7	10.9

[a] Average of two determinations. [b] Average of three determinations.

TABLE II.—*o*-THYMOTIC ACID ESTERS

Ester	% Yield[a]	Boiling Point, ° C.—Reported (12)	Boiling Point, ° C.—Found	Saponification Equivalent[b]—Calcd.	Saponification Equivalent[b]—Found[c]	Carbon and Hydrogen Analysis, %—Calcd.—C	Carbon and Hydrogen Analysis, %—Calcd.—H	Carbon and Hydrogen Analysis, %—Found[d]—C	Carbon and Hydrogen Analysis, %—Found[d]—H
Methyl	49.5	142/18.5 mm.	143/20 mm.	538	532	69.23	7.21	69.3	7.0
Ethyl	53.9	153/18.5 mm.	155/20 mm.	505	494	70.27	7.66	70.6	7.6
n-Propyl	50.3	...	165/20 mm.	475	464	71.19	8.05	71.4	8.0
n-Butyl	43.5	...	175/20 mm.	452	442	72.00	8.40	71.8	8.3

[a] Average of two determinations. [b] Expressed in mg. of potassium hydroxide. [c] Average of two determinations. [d] Average of two determinations.

TABLE III.—RATES OF ESTERIFICATION OF URSOLIC ACID ESTERS

Time of Exposure, min.	% Esterification—Methyl	% Esterification—Ethyl	% Esterification—*n*-Propyl	% Esterification—*n*-Butyl
0	0.0	0.0	0.0	0.0
15	1.2	0.0	0.0	0.4
30	4.3	0.6	2.5	0.4
45	10.5	1.9	3.8	4.7
60	16.6	7.2	6.4	6.1
75	35.2	20.4	15.5	14.5
90	69.1	45.6	35.0	34.1
105	76.8	57.6	42.8	39.7
120	78.3	61.6	45.4	43.9
135	79.9	64.2	48.0	48.1

TABLE IV.—RATES OF ESTERIFICATION OF *o*-THYMOTIC ACID ESTERS

Time of Exposure, min.	% Esterification—Methyl	% Esterification—Ethyl	% Esterification—*n*-Propyl	% Esterification—*n*-Butyl
0	0.0	0.0	0.0	0.0
15	0.0	0.0	0.0	0.0
30	0.3	0.2	0.6	0.0
45	6.3	3.9	5.1	2.9
60	12.3	12.1	10.3	9.4
75	20.3	21.0	17.1	16.1
90	30.2	30.7	27.5	24.1
105	44.2	45.6	43.9	38.6
120	48.8	47.9	46.9	44.5
135	50.8	49.4	46.9	45.9
150	51.5	50.1	47.7	45.9

Attempts to determine saponification equivalents of the esters were unsuccessful due to the resistance of these esters to even the most drastic conditions for hydrolysis.

Preparation of *o*-Thymotic Acid Esters.—The same method that was used to prepare ursolic acid esters was followed and the results were comparable, except that when the solvent was evaporated, the residue was liquid in all cases. These liquid esters were dissolved in ether and washed with 3% sodium hydroxide solution followed by washing with dilute hydrochloric acid solution. The ether was evaporated on a water bath and the remaining liquid was distilled *in vacuo*. The esters were colorless when distilled, but changed to pale yellow immediately afterward. Fractional distillation on a Todd column[2] failed to remove the yellow color. Again, only the straight chain esters could be synthesized (Table II). Carbon-hydrogen results and saponification equivalents by a method using diethylene glycol as the solvent (9) are shown also in this table.

Rates of Esterification of Ursolic Acid Esters.—The rate of esterification for each of the esters was determined under the same conditions that were used for their preparation. Weighed quantities of ursolic acid were exposed, using the proper alcohols, and aliquots were extracted at fifteen-minute intervals. These aliquots were titrated with standard sodium methoxide solution using thymol blue as the indicator. Sodium methoxide solution was used because reaction was too slow with other alkali solutions. The percentage of unesterified acid was calculated and the per cent of esterification was then apparent by difference. The results are shown in Table III.

Rates of Esterification of *o*-Thymotic Acid Esters.—The rate of esterification of each of the four esters was determined under the same conditions that were used for their preparation. Weighed quantities of *o*-thymotic acid were exposed, using the proper alcohols, and aliquot portions were extracted at fifteen-minute intervals and titrated with standard sodium hydroxide solution, using phenolphthalein as the indicator. The percentage of unesterified acid was calculated, and the per cent of esterification was then apparent by difference. The results of this study are given in Table IV.

Mechanism of Esterification Using Ultrasound.—One gram of ursolic acid was suspended in 100 ml. of ethanol. Two grams of methyl mercaptan and a drop of concentrated sulfuric acid were added. The mixture was exposed at 39.1 kc. and 45 ma.

[2] Todd Precision Fractionation Assembly, Todd Scientific Co., Springfield, Pa.

plate current for two hours. The odor of hydrogen sulfide was apparent after about one hour of the exposure. The solution was poured into water, and the white precipitate which formed was collected on a sintered-glass funnel. The compound was purified in the same manner as stated previously, yielding 0.452 Gm. (43.9%, of theoretical) of methyl ursolate, m. p. 168–170°. After a sodium fusion, no positive test for sulfur was obtained. A mixed melting point with a genuine sample of methyl ursolate gave no appreciable lowering of the melting point. The indication is that the acid acted as a hydrogen donor and not a hydroxyl donor in this reaction. Reaction of ursolic acid with ethyl mercaptan by the same procedure produced ethyl ursolate, m. p. 197–199°, further verifying the mechanism involved.

SUMMARY AND CONCLUSIONS

Low frequency ultrasound waves have been demonstrated to be effective in facilitating the direct esterification of *o*-thymotic and ursolic acids, both of which are sterically hindered.

Straight chain esters of the acids, using alcohols having up to four carbon atoms, were prepared. Branched chain esters of these acids could not be prepared even after prolonged periods of exposure and increased plate current. This is believed to be due to the bulk of the entering group.

Data showing the rates of esterification of the acids are reported.

The mechanism of the reaction using ultrasound was established to be the donation of hydrogen by the carboxyl group rather than the donation of hydrogen by the alcohol.

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Studies on the Stability of Filipin I*

Thermal Degradation in the Presence of Air

By JAMES E. TINGSTAD and EDWARD R. GARRETT

The loss of biological activity of crystalline filipin has been statistically correlated with the loss of spectrophotometric absorbance at 355 mμ. Filipin is oxidatively destroyed in air by an apparent second-order reaction similar to the one involved in the aerobic degradation of fumagillin. The degradation product(s) interfere slightly with the U. V. assay, and correction factors are applied. The rate constants for the reaction at 70, 60, 50, and 37° have been evaluated and the apparent heat of activation calculated. Half lives at 30, 25, and 4° have been estimated. Preliminary experiments show that filipin is at least 50 to 100 times more stable under nitrogen than in air. A new empirical formula, $C_{35}H_{58}O_{10}$, determined on especially purified filipin is given.

FILIPIN, A POTENT antifungal agent isolated from *Streptomyces filipinensis* by Whitfield and co-workers (1), is a neutral conjugated polyene with an empirical formula of $C_{32}H_{54}O_{10}$.[1]

Filipin strongly absorbs ultraviolet light, with maxima at 322, 338, and 355 mμ (Fig. 1, curve A). Whitfield and associates reported (1) that filipin is susceptible to oxidation and that the loss of absorbance at 338 and 355 mμ closely parallels the loss of biological activity. This is to be expected since its general structure resembles that of another polyene antibiotic, fumagillin; the degradation of which was studied by Garrett and Eble (2, 3, 4).

In order to determine the normal shelf life of the drug if protected from light, the thermal, aerobic, nonphotolytic degradation of crystalline

* Received August 21, 1959, from the Upjohn Co., Kalamazoo, Mich.
Presented to the Scientific Section, A. Ph. A., Cincinnati meeting, August 1959.
[1] In a previous publication (1) the empirical formula was given as $C_{35}H_{58}O_{10}$. Recent analyses, by W. A. Struck and associates of these laboratories, of material vigorously purified by G. B. Whitfield, also of these laboratories, confirm the new formula.

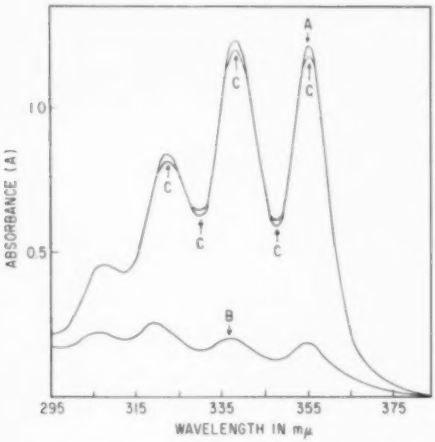

Fig. 1.—Ultraviolet absorption spectra for *A*, undegraded filipin; *B*, filipin exposed to air at 70° for forty-eight hours; and *C*, filipin under nitrogen exposed to the same conditions. All are at 10 mcg./ml. concentration in absolute methanol.

filipin was investigated. In this kinetic study a spectrophotometric assay was used, and the absorbance at 355 mμ was statistically correlated with biological activity.

EXPERIMENTAL

Correlation of Spectrophotometric Data with Biological Activity.—The calculated concentration[2] of thermally degraded filipin was statistically correlated with the *S. pastorianus* assay procedure of Burch and Sokolski (6).[3] The spectrophotometric data used for this correlation were those obtained in the degradation rate study at 70°.

The statistics of correlation are represented by the plot in Fig. 2. The biological assay value (P) is given in terms of micrograms of filipin activity per milligram of sample as compared with a standard. The concentration (C_f) of filipin as calculated from the observed absorbance at 355 mμ is defined as the fraction of the total material that is undegraded filipin.

The regression equation is P (mcg./mg.) = $mC_f + b$ where m and b are slope and intercept, respectively. The actual equation is $P = 967C_f + 36$. The 95% confidence limits of the slope and intercept are ±148 and ±104, respectively. A slope of 1,000 mcg./mg., which signifies a 1:1 ratio between the variables, and an intercept of zero can readily be accepted from these estimates of error. Thus it can be concluded that excellent correlation exists between spectrophotometric and biological assays. If the variation about the regression is assumed to be due to error in the bioassay and not a function of the magnitude of the assay, the standard deviation of the *S. pastorianus* assay may be estimated as ±57 mcg./mg. and the 95% confidence limits as ±126 mcg./mg.

Degradation Rate Studies.—One-milliliter aliquots of a 5 mg./ml. methanolic solution of filipin were pipetted into 10-ml. amber vials and the solvent removed by vacuum distillation. The unsealed vials were then placed in covered 1-pound amber ointment jars and immersed in constant temperature baths at 37 ±3, 50.0 ±0.5, 60.5 ±0.5, and 70.0 ±0.5°. The ointment jars were used (rather than just sealing the small vials) to provide a relatively large air space, thus keeping the oxygen concentration essentially constant. It was necessary to seal the system in some way because the samples were kept in a lighted room, and it was desired to keep the reaction nonphotolytic. Samples were periodically removed from the baths, diluted with absolute methanol to 10 mcg./ml., and assayed at 355 mμ using a Beckman model DU spectrophotometer.

In a preliminary experiment designed to study the stability of crystalline filipin in the absence of air, the same procedure was followed as for the air samples except that each vial was sealed with a rubber stopper and aluminum cap, then evacuated and filled with nitrogen by piercing the stopper with a hypodermic needle and alternately applying suction and nitrogen under pressure. In this manner the sealed vials were flushed with nitrogen three times and stored at 70°.

RESULTS AND DISCUSSION

Curve A in Fig. 1 represents the spectrum of undegraded filipin, curve B the spectrum of filipin exposed to air at 70° for forty-eight hours, and curve C the spectrum of filipin exposed to nitrogen at 70° for forty-eight hours. It is readily apparent that the drug is much more stable in the absence of air.

In the aerobic degradation study, it was observed that the absorbance at 355 mμ did not go to zero in the expected length of time. Instead, it reached an asymptotic value which was different for each temperature (Fig. 3). When this value was reached, the absorbance declined at a rate much slower than the original.

The asymptotic values for the absorbances at the various temperatures are as follows:

Temperature °C.	Asymptotic Value, A_{t_∞}
70.0	0.07
60.5	0.10
50.0	0.20
37.0	0.27

The above data indicated that the first degradation step(s) resulted in a product(s) which absorbed some light at 355 mμ; this material then degraded further to give a nonabsorbing product(s). The fact that the asymptotic value was different for each temperature (it was practically zero at 70°) indicated that different degradation products, each having a different absorptivity, were formed at the various reaction temperatures. This agrees with findings on fumagillin reported by Garrett and Eble (4).

[2] The concentration of filipin was calculated from the observed absorbance (5) at 355 mμ in absolute methanol at 0.01 mg./ml. concentration. Theory and calculations are given under *Results and Discussion*.

[3] The authors are indebted to M. Burch and co-workers of the Microbiology Department of The Upjohn Co. for the biological assays.

On the basis of the above postulates it became necessary to calculate the actual concentration of filipin at any time (t). This would eliminate most of the error in the observed absorbance due to the contribution of the degradation products(s).

Let A_f = absorbance of filipin in methanolic solution (using a 1-cm. cell); a_f = absorptivity of filipin in methanol; C_f = concentration of filipin in the crystal lattice of the solid (defined as the fraction of total solids that is undegraded filipin); and v = volume of methanol in which C_f is dissolved to give absorbance A_f.

Then

$$A_f = a_f C_f / v \qquad \text{(Eq. 1)}$$

Similarly

$$A_x = a_x C_x / v \qquad \text{(Eq. 2)}$$

where x is the degradation product (v is constant throughout the calculations).

At any time (t) let

$$C_f + C_x = 1 \qquad \text{(Eq. 3)}$$

and

$$A_t = A_f + A_x \qquad \text{(Eq. 4)}$$

where A_t is the observed absorbance at any time (t).

Then

$$A_t = a_f C_f / v + a_x C_x / v \qquad \text{(Eq. 5)}$$

$$vA_t = a_f C_f + a_x(1 - C_f) \qquad \text{(Eq. 6)}$$

$$vA_t = a_f C_f + a_x - a_x C_f \qquad \text{(Eq. 7)}$$

$$vA_t - a_x = C_f(a_f - a_x) \qquad \text{(Eq. 8)}$$

$$C_f = (vA_t - a_x)/a_f - a_x \qquad \text{(Eq. 9)}$$

Equation 1 reduces to

$$a_f = vA_{t_0} \qquad \text{(Eq. 10)}$$

when $t = 0$, since then $C_f = 1$, $C_x = 0$, $A_x = 0$, and $A_t = A_f$. Similarly, Eq. 2 reduces to

$$a_x = vA_{t_\infty} \qquad \text{(Eq. 11)}$$

when $t = \infty$, since then $C_f = 0$, $C_x = 1$, $A_f = 0$, and $A_t = A_x$. The term t_∞ is defined as the time when filipin is completely degraded to x and x has not yet started to degrade. It follows that

$$C_f = \frac{vA_t - vA_{t_\infty}}{vA_{t_0} - vA_{t_\infty}} \qquad \text{(Eq. 12)}$$

and

$$C_f = \frac{A_t - A_{t_\infty}}{A_{t_0} - A_{t_\infty}} \qquad \text{(Eq. 13)}$$

Three assumptions are made here: (a) that filipin and its degradation products follow the Beer-Lambert Law; (b) that filipin is completely degraded to x, and (c) that the rate of degradation of x is insignificant compared with that of filipin. The first assumption is supported by the results of the bio-spectrophotometric correlation study.

The second introduces some error, since the aerobic degradation of a polyene may yield several products, each at a different stage of oxidation. However, for purposes of calculation it is convenient and reasonable to take a_x as the average absorptivity of all the degradation products whose concentration in the crystal lattice is C_x. This assumption is supported by the fact that equations resulting from it give the best fit to the data.

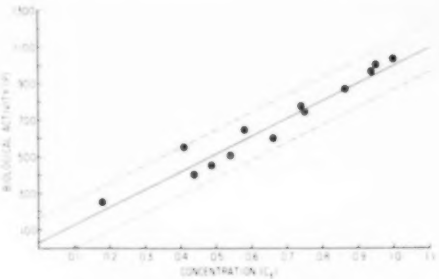

Fig. 2.—Plot of biological activity (P) in mcg./ml. *vs.* calculated concentration (C_f) of filipin as determined from U. V. assay at 355 mμ. The solid line is of best statistical fit and the dashed lines are 95% confidence limits of a predicted P from a specific C_f.

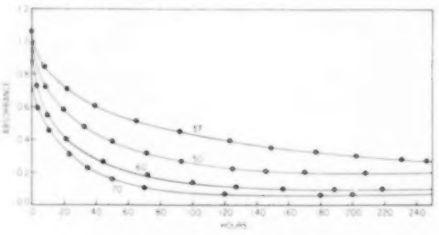

Fig. 3.—Plot showing the absorbances reaching asymptotic values, different for each temperature, as the reaction nears completion.

The third assumption is supported by the behavior of the absorbance values during the kinetic studies and by the fact that the deviations from the straight line plot (Fig. 4) do not appreciably increase as the reaction proceeds.

When the data were plotted, it was evident that the reaction was not zero or first order. Plots of the concentration of filipin and log of the concentration against time did not yield straight lines. Plotting the reciprocal of the concentration *vs.* time (Fig. 4) resulted in a straight line, indicating that the degradation was most probably second order with respect to filipin. The mechanism of the reaction is probably very similar to that proposed for the aerobic degradation of fumagillin by Garrett and Eble (4).

The reaction rate constants at the various temperatures, calculated from the slopes of the corresponding lines in Fig. 4, are as follows:

Temperature, °C.	Reaction Rate Constants (k), hr.$^{-1}$
70.0	0.178
60.5	0.126
50.0	0.081
37.0	0.042

An Arrhenius plot of the log of the reaction rate constants obtained in Fig. 4 against the reciprocals

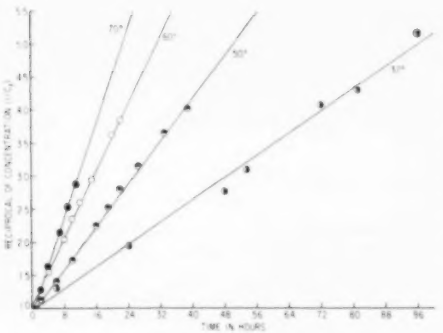

Fig. 4.—Plot illustrating the second order thermal degradation of crystalline filipin in the presence of air. The reciprocal of C_f is plotted against time in hours for several temperatures.

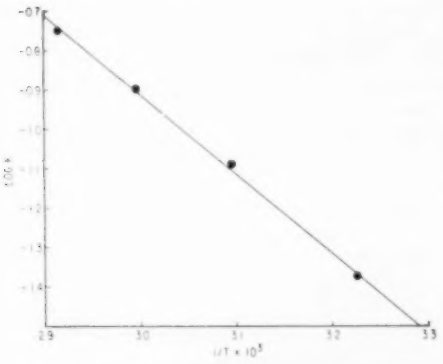

Fig. 5.—Arrhenius plot of the logarithm of the rate constant (k) for the thermal oxidation of filipin against the reciprocal of the absolute temperature (T).

of the absolute temperatures is shown in Fig. 5. The equation of best fit is

$$\log k = -2028/T + 5.17$$

The standard deviation of a predicted log k at a given temperature is 0.014 and that of the slope is 63. The apparent heat of activation is 9.3 Kg. cal./mole. This heat of activation is very close to that for the aerobic degradation of fumagillin (4)

The half lives of filipin at 30, 25, and 4° were estimated to be thirty-three, forty-three and one hundred and forty-one hours, respectively.[4] At first glance, these data indicate that storage of the drug would be a major problem.

However, the environment to which the filipin was exposed in this study was much more severe than that which would exist under normal storage conditions. Removal of the methanol by vacuum distillation left the crystalline filipin with a much larger amount of exposed surface area than would be normally present. Furthermore, the oxygen supply was essentially unlimited, whereas a bottle nearly filled with drug and kept tightly closed for long periods of time (normal storage conditions) would contain a limited amount of oxygen.

These postulations are supported by results of studies on crystalline filipin reported by Whitfield (7) who found a 25% loss of potency at 25° and 13% loss at 4° after one year's storage in a closed amber bottle.

Preliminary experiments indicate that filipin is 50 to 100 times more stable in the absence than in the presence of air (see Fig. 1). The half life of filipin in air at 70° is about six hours. The apparent half life of filipin under nitrogen at 70° is in excess of seven hundred and twenty hours. Results from the nitrogen experiments are not accurate due to leakage of air into some of the sealed vials, and the experiments will have to be repeated. However, air leakage would cause the apparent half life under nitrogen to be shorter than it actually is; therefore the true half life is probably longer than the one determined in these experiments.

SUMMARY

1. Spectrophotometric absorbances at 355 mμ give good indication of the progress of the aerobic degradation of crystalline filipin. Absorbances have been statistically correlated with biological assays.

2. Crystalline filipin is rapidly destroyed by heat in the presence of air. The reaction is oxidative and appears to be second order.

3. The mechanism of the reaction is probably similar to the one responsible for the aerobic degradation of fumagillin.

4. Preliminary experiments indicate that filipin is at least 50 to 100 times more stable under nitrogen than it is in air.

5. The empirical formula for filipin is now given as $C_{35}H_{58}O_{10}$.

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[4] The half lives were calculated from the equation $t_{1/2} = 1/kC_{f_0}$ where C_{f_0} is the initial concentration of filipin in the solid material. It can be seen that $t_{1/2}$ is a function of initial concentration, and that half lives in a second-order reaction have little meaning unless initial concentration is specified. However, in this experiment it is assumed that the starting material was 100% filipin; C_{f_0} can then be normalized to 1, and the equation reduces to $t_{1/2} = 1/k$.

The Complexing Tendencies of Cyanocobalamin With Inorganic Compounds*

Heteromolybdates and Heavy Metal Chlorides

By RUTH N. HAVEMEYER† and TAKERU HIGUCHI

The interactions of vitamin B_{12} with numerous inorganic compounds have been investigated. Nine of the compounds studied yielded slightly soluble adducts with cyanocobalamin. These were phosphotungstic and phosphomolybdic acid, the sodium, nickel, and manganese salts of phospho-12-molybdic acid, and the chlorides of gold, platinum, and palladium. Several of the reagents caused a change in the absorption spectrum of the vitamin in the system. This effect was found to be a function of the concentration of the reagent, but independent of hydrogen ion concentration.

THE ROLE OF COMPLEX FORMATION of cyanocobalamin (vitamin B_{12}) as affects its absorption in the gastrointestinal tract has received much attention. There have been reports of the apparent interaction tendencies of the vitamin with mucoproteins, sugar alcohols, peptides, and other organic compounds (1–4).

In earlier work from these laboratories, studies were carried out on the association tendencies of vitamin B_{12} with several classes of organic compounds. Szulczewski and Higuchi (5) studied the complex formation with sugars, sugar alcohols, and phenolic compounds. Mouri and Higuchi (6) interacted the vitamin with various carboxylic acids, amines, amides, and diols. The investigation reported at the present time is concerned with the binding of the vitamin with heteropoly acids and their salts, together with its interactions with some metal chlorides.

Studies of this nature were considered of pharmaceutical interest from two standpoints. It was hoped firstly that they may provide some insight into the influence of various additives upon the rate and efficiency of absorption of the vitamin when taken orally. Secondly, it was thought that some dosage form of the depot type might be based on the discovery of some extremely insoluble complexes formed from cyanocobalamin. It would be pharmacologically desirable to have available dosage forms of vitamin B_{12} which would permit its slow and controlled release into the system. One possible way to realize this might be to form slowly soluble molecular addition compounds, or complexes, suitable for parenteral use.

Such interactions may reasonably be expected. The vitamin B_{12} molecule (Fig. 1) includes several groups which might conceivably act as complexing sites; these are the amide functions, the orthophosphate moiety, the cyano-cobalt system, and the nucleotide portion of the molecule. Interactions of some of these functional types are well-known. For example, orthophosphates complex strongly with ferric ion (7), and simple amides such as acetamide and benzamide form crystalline addition compounds, which can be isolated from aqueous solution, with gold and platinum chlorides (8, 9). Many inorganic compounds function as protein precipitants (10, 11), and some of these may well be expected to act similarly upon the cyanocobalamin structure.

Since poorly soluble or slowly soluble adducts were desired, some substances of high molecular weight were employed as the complexing agents. Other compounds, such as gold chloride, were

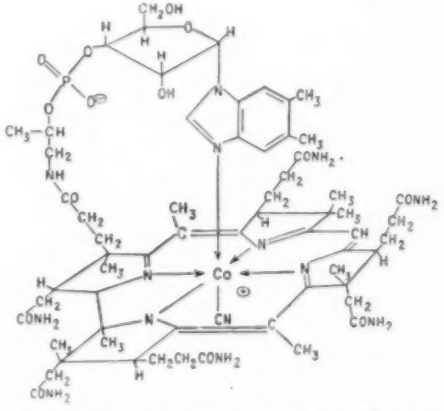

Fig. 1.—The structure of cyanocobalamin. Reprinted by permission, from p. 19 of "Vitamin B-12 und Intrinsic Factor," H. C. Heinrich, editor.

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chosen on the basis of known interactions with pertinent functional groups. Still others were selected for study in an attempt to separate out specific ion effects. In addition, such studies may yield some information as to the sites of interaction.

EXPERIMENTAL

Reagents.—*Heteromolybdates.*—Sodium-phospho-12-molybdate, sodium-silico-12-molybdate, sodium-2-phospho-18-molybdate, Climax Molybdenum Co.; phosphotungstic acid, Eastman Kodak; phosphomolybdic acid, Merck and Co.; manganese-phospho-12-molybdate, nickel-phospho-12-molybdate.

The first five compounds were recrystallized from hot water. The last two were not commercially available and were synthesized by the method of Arnfeld, as follows (12): Freshly precipitated nickelous (or manganous) phosphate was suspended in hot water. To this was added, in small portions, the stoichiometric quantity of molybdenum trioxide which had been recently heated to glowing in a porcelain crucible. The suspension was heated gently and small quantities of water were added until all of the solids dissolved. This solution was concentrated over sulfuric acid and yielded greenish-yellow crystals. The first crystals to separate, those of the phospho-12-molybdic acid, were filtered off in order to separate them from the salt of the 9-acid, which are the last to separate. An aqueous solution of the 12-acid salt, when treated with ammonium or potassium chloride, yields a precipitate. A solution of the 9-acid salt shows no reaction with either of these reagents.

Chlorides.—Aluminum, ammonium, barium, cadmium, cobaltous, cupric, ferric, hexamino-cobalt, lithium, lead, manganous, mercuric, nickelous, sodium, zinc, auric, platinic, palladous, and potassium gold chlorides.

All but the last four compounds were recrystallized from hot water. These four chlorides were used as supplied by the manufacturers; they were not recrystallized because of their hygroscopicity and because of the small quantities available.

Procedure.—The method of study was phase solubility analysis, using a water bath thermostatted at 25 ± 0.05°. The method and the apparatus have been described previously (13, 14).

The crystalline vitamin B_{12} was weighed into 2-cc. vials and sufficient water was added to give a concentration of 20 mg. B_{12}/Gm. water. Sufficient reagent was then added to give the desired concentrations. After a twenty-four-hour equilibrium period, the supernatant liquid was withdrawn through medium-porosity sintered-glass disks. These aliquots were placed in tared 10-ml. volumetric flasks, and the weights of the aliquots were determined. The diluted samples were analyzed for B_{12} content by measuring their absorbance at 550 mμ, using the Cary recording spectrophotometer model 11MS.

All solutions containing B_{12}, in free or combined form, were saved for recovery of the vitamin. The pooled solutions were first lyophilized, and the dry powder obtained was redissolved to give a concentrated solution. This concentrate was then treated by the method of Bernhauer and Friedrich (15) to obtain a purified aqueous solution, and the crystalline vitamin was finally precipitated by addition of 90% acetone. The acetone used was first purified by refluxing it for several hours with potassium permanganate and potassium carbonate, and was then distilled. The reclaimed vitamin was checked for purity spectrophotometrically.

Sample Calculation to Determine the B_{12} Content of the Diluted Aliquots.—(*a*) (Absorbance) (volume of final soln./absorptivity) = mg. B_{12}/10 ml., (*b*) (mg. B_{12}/10 ml.) (1/weight of aliquot) = mg. B_{12}/Gm. (*c*) (mg. B_{12}/Gm.) ÷ (molecular weight of B_{12}) = 10^{-6} mole B_{12}/Gm.

RESULTS

Heteromolybdates.—Figure 2 shows the curves for those interactions which resulted in only an increase in the solubility of cyanocobalamin; Figs. 3 and 4 give the complexing curves for those compounds which interacted with B_{12} to give an insoluble form.

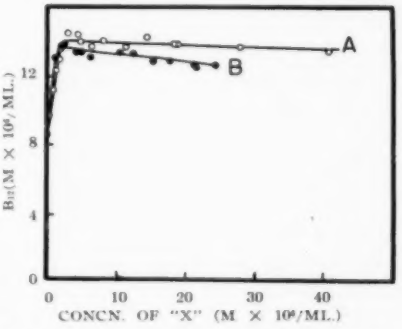

Fig. 2.—A, sodium-silico-12-molybdate; B, sodium-2-phospho-18-molybdate.

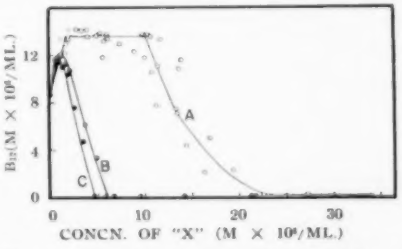

Fig. 3.—A, sodium-phospho-12-molybdate; B, nickel-phospho-12-molybdate; C, manganese-phospho-12-molybdate.

The scatter observed may be the cumulative result of the experimental errors, the principal one of which is probably the weighing error. It is not the result of insufficient equilibration, since the same effect was observed when the equilibrium time was extended to forty-eight hours.

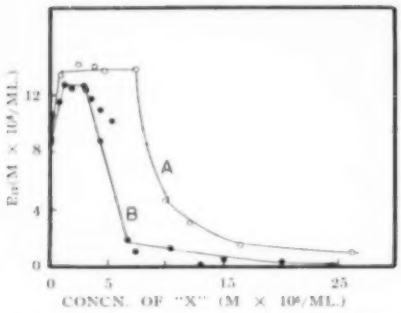

Fig. 4.—A, phosphotungstic acid; B, phosphomolybdic acid.

The data for the compounds which gave insoluble reaction products are given in Table I. The approximate stoichiometries of the solid complexes were calculated in three ways: (*a*) from the initial slope of the descending portion of the curve; (*b*) from the amount of complexing agent necessary to remove all of the vitamin from the system, determined by extrapolating the downcurve of the plots to the *x*-axis. The value, total B_{12}/total agent, is the ratio of the concentration of the vitamin to this value; and (*c*) by spectral analysis, accomplished by calculating the concentration of B_{12} from the absorbance of a solution of 1 mg. of the solid in 25 ml. of water.

Of the three methods, the spectral analysis of the solid is probably the most reliable. Since the other two are based upon calculations from the phase diagrams they have rather large inherent errors in them. The downslopes, for example, are only best-fitting lines by visual (not mathematical) analysis, and in at least one of the curves there is considerable scatter. Also, because of the steepness of the downslopes, it is often difficult to obtain precise numerical values therefrom.

Chlorides.—Of the nineteen chlorides studied, platinic, auric, palladous, and potassium gold chlorides gave insoluble reaction products with cyanocobalamin (Figs. 5 and 6, Table II). Mercuric chloride is the only one which did not cause an initial increase in the solubility of the vitamin (Fig. 7).

Gold, potassium gold, and palladium chlorides caused a shift in the spectrum of B_{12} in the supernatant liquid. In the case of the gold salts, the two maxima at 550 mμ and 520 mμ blended into one maximum at about 525 mμ. This change is a function of concentration of the salt added but is independent of hydrogen ion concentration, as shown by a pH-dependence study using HCl.

With palladous chloride, also, the spectral shift was dependent upon the concentration of the chloride added. However, the spectral change observed in this system was more complicated, several changes in spectrum being noted with changing palladium concentration.

TABLE I—THE HETEROMOLYBDATES

Reagent	Approximate Molar Ratio (B_{12}:Agent) of Solid Complex Method of Calculation: Downslope	Total B_{12} / Total Agent	Spectral Analysis
Na-phospho-12-molybdate	10:3 (?)	1:1	1:1
Mn-phospho-12-molybdate	7:2	7:2	6:1
Ni-phospho-12-molybdate	3:1	5:2	5:2
Phosphomolybdic acid	2.7:1	2:1	1.7:1
Phosphotungstic acid	6:1 (?)	8:5 (?)	4:1

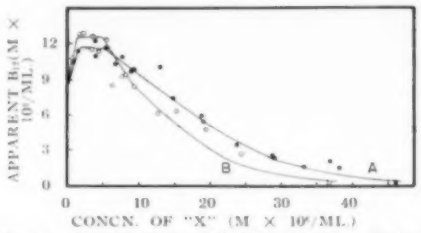

Fig. 5.—A, potassium gold chloride; B, palladium chloride.

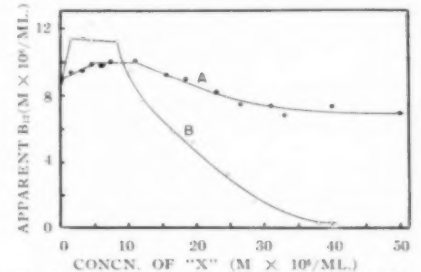

Fig. 6.—A, platinum chloride; B, gold chloride.

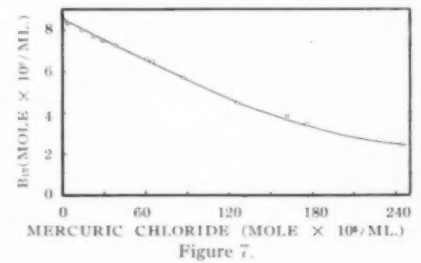

Figure 7.

DISCUSSION

Heteromolybdates.—The heteropoly anions are composed of molybdenum oxide groups which surround a central atom such as potassium, tungsten, or silicon. For example, in the phospho-12-molybdates, such as $Na_3(PMo_{12}O_{40})$, there is a PO_4 tetrahedron surrounded by twelve MoO_6 octahedra (Fig. 8). These twelve octahedra are joined together by the sharing of oxygen atoms at the corners. In the hetero-12-molybdates, there are twelve molybdenum atoms to one central atom; in the hetero-2,18-molybdates, there are eighteen molybdenum atoms to two central atoms. The structures of the two complex acids used are similar; each consists of a

TABLE II.—THE TRANSITION METAL CHLORIDES

Reagent	% Change in B_{12} Solubility[a]	Approx. Stoichiometry of Solid Complex (B_{12}:Agent) Method of Calculation: Downslope of Plot	Total B_{12} / Total Agent	Spectral Analysis of Precipitate	Upper limit of Reagent Concn. (10^{-6} Mole/ml.)
$HAuCl_4$	−100	3:2	1:1	incompletely soluble[b]	..
H_2PtCl_6	− 16	1:7	1:5	2:3	..
$KAuCl_4$	−100	2:5	1:2	incompletely soluble	..
$PdCl_2$	−100	1:1	4:5	insoluble[c]	..
$HgCl_2$	− 71[d]	..	..	..	..
$PbCl_2$	+0.6	..	..	..	30
NaCl	+ 4	..	..	..	5480
LiCl	− 9	..	..	..	90
$CoCl_2.6H_2O$	+ 15	..	..	..	44
$NiCl_2.6H_2O$	+ 15	..	..	..	49
$CdCl_2$	+ 15	..	..	..	114
$MnCl_2.4H_2O$	+ 18	..	..	..	50
$CuCl_2.2H_2O$	+ 20	..	..	..	74
$BaCl_2$	+ 23	..	..	..	115
$ZnCl_2$	+ 30	..	..	..	110
NH_4Cl	+ 30	..	..	..	656
$AlCl_3$	+ 30	..	..	..	46
$[Co(NH_3)_6]Cl_3$	+ 53	..	..	..	173
$FeCl_2.6H_2O$	+ 65	..	..	..	58

[a] + Indicates increase in solubility and − indicates decrease in solubility, calculated at reagent concentration in column 6. [b] One milligram incompletely soluble in 25 ml. of water. [c] One milligram insoluble in 25 ml. water. [d] See Fig. 7.

PO_4 tetrahedron surrounded by six XO_6 octahedra, where X is molybdenum or tungsten. These compounds are generally highly hydrated and may contain as many as forty molecules of water. Some of these water molecules are intimately bound in the crystal lattices and others are apparently zeolytic, i. e., they are lost on heating because they are not part of the crystal structure (16, 17).

The molecular weights of the heteromolybdates range from 2,000 to 4,000. Because of the complexity of these anions, there is still much confusion as to their structures and bond types. Another complication is that in solution there is an equilibrium between several of the anionic species.

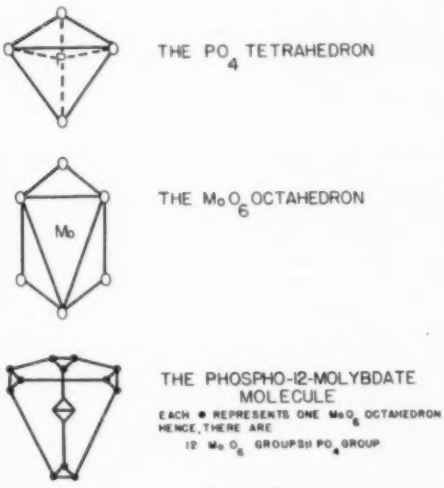

Figure 8.

From the complexing data, it would appear that for the several compounds studied, the ratio of 12 Mo: 1 P in the structure of the heteromolybdate is required to obtain an insoluble or slowly soluble adduct with the vitamin.

Chlorides.—The first chlorides studied were those of gold and platinum, since it was known that they react with acetamide groups (8, 9). The results of these interactions with vitamin B_{12} led to the investigation of other chlorides.

The spectrum change observed as a result of the interactions of the vitamin with gold and potassium gold chlorides is similar to that reported by Kaczka, *et al.*, on work done with various B_{12} analogs, in which the cyanide group coordinated to the cobalt in the B_{12} molecule was replaced with hydroxide, chloride, bromide, sulfide, sulfate, nitro, and cyanate groups (18). Hence, it is possible that cyanocobalamin is no longer present in solution. Possible causes of such spectral shifts may be replacement of the cyanide by other anions, interaction of the cation with the molecule in such a way as to disturb the conjugated bond system which is responsible for the characteristic absorption spectrum of vitamin B_{12}, or oxidation-reduction reactions due to the presence of the auric ion. The shifted peak obtained in this study, 525 mμ, could conceivably correspond to that of cyanatocobalamin (18).

A photometric study was undertaken in which a saturated solution of gold chloride or palladium chloride was added dropwise to a rapidly stirred vitamin B_{12} solution. The titration flask was connected to a Corex cell (19), and the spectrum of the resultant solution was recorded after each addition of the metal chloride. In each case, the reaction which resulted in the changing spectrum was found to be time dependent.

The spectral changes observed in the palladium chloride-cyanocobalamin system seem to indicate that there are several complex species forming.

We were unable to determine isosbestic points in those samples in which the agent was present in concentrations low enough to form soluble species, and it may be that at all reasonable concentrations of palladium chloride there are more than two species in equilibrium. Since the palladium salt used, palladous chloride, is not palladium in its highest oxidation state, a more complex oxidation-reduction reaction may be occurring. It may be for this reason that the spectral shift is not constant.

The infrared spectra of several of the insoluble reaction products were inconclusive as regards the nature of the reactions taking place.

The decrease in solubility observed with mercuric chloride and vitamin B_{12} may be the result of a salting out effect or a medium effect upon the absorption spectrum. The latter is perhaps a more feasible explanation, since the concentrations of mercuric chloride used were very low. It seems reasonable to expect that if there were complex formation between these compounds, then an initial plateau region would result until all of the solid excess B_{12} in the system had reacted.

The majority of the metal chlorides employed in this study resulted in an increase in the solubility of the cyanocobalamin (Table II), but without a subsequent decrease. These compounds may be of value as additives to aid in the increased absorption of vitamin B_{12} preparations, or they may prove to be useful in the stabilization of the vitamin in solution.

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Antioxidants. The Microdetermination of Hydroquinone*

By MOHAMED Z. BARAKAT, SAAD K. SHEHAB, and ABDEL MAKSOUD ABDALLA

A new titrimetric method for the microdetermination of hydroquinone is described. This method is recommended for the assay of fats and oils, photographic developers, and ether anesthetics. The assay is carried out within limits of 0.5 to 2 mg. of hydroquinone. The procedure is quite simple and rapid but yet shows relatively high accuracy over the suggested range. The experimental error does not exceed ±2 per cent.

HYDROQUINONE is an effective antioxidant for fats and oils (1–5). In tropical districts, hydroquinone is used as a preservative for ether anesthetics (6). Furthermore, hydroquinone is a prominent constituent in photographic developers (7). Hence, our interest in the microdetermination of hydroquinone.

Previously, various methods have been reported (8–12) for the determination of hydroquinone. Of these, the titrimetric methods are used extensively because of their rapidity and simplicity. So far as we are aware, certain de-

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fects are involved in the previously known volumetric methods (13–16). Moreover, the reported experimental error (17) is outstanding, being ±20 per cent.

The present investigation deals with a new easy, rapid, and accurate titrimetric method for the microdetermination of hydroquinone.

EXPERIMENTAL

Equipment.—A microburet of 5-ml. capacity, graduated in hundredths of a milliliter; graduated pipets of 1-, 2-, and 5-ml. capacity; Erlenmeyer flasks of 25-, 50-, 100-, and 250-ml. capacity.

Reagents.—Aqueous potassium iodide solution, 4% w/v; aqueous starch solution, 1% w/v; a saturated aqueous sodium bicarbonate solution; aqueous N-bromosuccinimide solution, 0.1% w/v, which may be serially diluted 10 times as required.

Validity of Reaction for Quantitative Estimation.—The authors decided to verify the action of N-bromosuccinimide on hydroquinone (18) from a quantitative point of view. Practically it was found that one molecule of hydroquinone was oxidized by one molecule of N-bromosuccinimide. The reaction was also valid to estimate hydroquinone in concentrations as low as 55 γ per 5 ml. of solution.

Procedure.—Into a 50-ml. Erlenmeyer flask, a known volume of the unknown hydroquinone solution is introduced, i. e., 1 ml. contains 1 mg. of hydroquinone. Then an equal volume of saturated sodium bicarbonate solution, 5 ml. of 4% potassium iodide solution, and 10 drops of starch solution as an indicator are added. The aqueous N-bromosuccinimide solution (0.1%) is introduced into the microburet and is allowed to run, drop by drop, into the hydroquinone solution, with continuous shaking. The end point is reached when the last drop of the N-bromosuccinimide solution added produces a permanent blue color in the hydroquinone solution (Table I).

A 0.1% hydroquinone solution was estimated simultaneously, with 0.01 *N* iodine (Table II).

METHOD OF ASSAY

Fats and Oils.—For the estimation of hydroquinone in fats or oils, the following method is recommended. A known weight or volume of the fat (10 Gm.) or oil (10 ml.), respectively, containing 0.5 to 2 mg. of hydroquinone, is dissolved in 150 ml. of ether; in case of oils the pipet being washed twice with ether. Then the procedure for estimation of pure hydroquinone solutions is followed but the end point is violet.

This method has been successfully applied to butter, lard, and cod liver oil. Hydroquinone (0.1 Gm.) was dissolved in ether (100 ml.) in a stoppered standard flask and 20 ml. of this ethereal solution (containing 20 mg. of hydroquinone) was added to 100 Gm. of freshly melted butter or lard and to 100 ml. of cod liver oil. The ether was evaporated and each sample was allowed to cool. Consequently, 10 Gm. of butter or lard and 10 ml. of cod liver oil should contain 2 mg. of hydroquinone (Table III).

TABLE I.—ESTIMATION OF PURE HYDROQUINONE SOLUTIONS BY 0.1% NBS[a]

Hydroquinone content = $V \times C \times 110/178$ (mg. or mcg.) where V = volume of N-bromosuccinimide solution and C = concentration of N-bromosuccinimide solution either in mg. or mcg.

Hydroquinone Soln., %	Volume, ml.	Content, mg.	0.1% NBS Used, ml.	Found, mg.	Error, %
0.5	1	5	8.15	5.04	0.80
	2	10	16.20	10.01	0.10
0.4	1	4	6.45	3.99	0.25
	2	8	13.00	8.03	0.38
0.3	1	3	4.85	3.00	..
	2	6	9.90	6.12	2.00
	3	9	14.50	8.96	0.44
0.2	1	2	3.20	1.98	1.00
	2	4	6.50	4.02	0.50
	3	6	9.70	5.99	0.17
	4	8	13.00	8.03	0.38
	5	10	16.20	10.01	0.10
0.1	10	10	16.00	9.89	1.10
	9	9	14.60	9.02	0.22
	8	8	13.00	8.03	0.38
	7	7	11.40	7.04	0.57
	6	6	9.90	6.12	2.00
	5	5	8.15	5.04	0.80
	4	4	6.55	4.05	1.25
	3	3	4.85	3.00	..
	2	2	3.25	2.01	0.50
	1	1	1.60	0.99	1.00

[a] N-Bromosuccinimide solution.

TABLE II.—COMPARATIVE ANALYSIS BY PROPOSED METHOD AND BY IODINE

0.1% Hydroquinone Soln., ml.	Content, mg.	Found by 0.1% NBS, mg.	Error, %	Found by 0.01 *N* Iodine, mg.	Error, %
10	10	9.98	0.20	10.01	0.10
8	8	8.03	0.38	7.98	0.25
6	6	6.05	0.83	6.05	0.83
4	4	3.96	1.00	4.13	3.25
2	2	2.04	2.00	2.09	4.50
1	1	1.01	1.00	1.045	4.50

TABLE III.—DETERMINATION OF HYDROQUINONE IN BUTTER, LARD, AND COD LIVER OIL

Sample	Weight of Fat or Volume of Oil, Gm. or ml.	Hydroquinone Content, mg.	0.1% NBS Used, ml.	Hydroquinone Found, mg.	Error, %
Butter	10	2	3.20	1.98	1.00
Lard	10	2	3.18	1.97	1.50
Cod liver oil	10	2	3.30	2.04	2.00
	5	1	1.60	0.99	1.00
	2.5	0.5	0.80	0.49	2.00

Photographic Developers.—One example is sufficient to illustrate the validity of the assay method, e. g., the following solution:

Metol	0.1 Gm.
Sodium sulfite	1.3 Gm.
Hydroquinone	0.3 Gm.
Sodium carbonate	2.6 Gm.
Potassium bromide	0.1 Gm.
Water *ad*	100 ml.

Method.—To 2 ml. of the developer add 20 ml. of 10% barium chloride solution, shake well, and filter after ten minutes (away from sunlight). To the filtrate apply the same procedure for pure hydroquinone solutions. Only the end point is violet.

Developer, ml.	Hydroquinone Content, mg.	0.1% NBS Used, ml.	Hydroquinone Found, mg.	Error, %
2	6	9.90	6.12	2.00

Ether Anesthetics.—The same procedure for pure hydroquinone solutions is adopted. The results of estimating 1% w/v of hydroquinone in ether and a serial dilution (10 times) of it are given in Table IV.

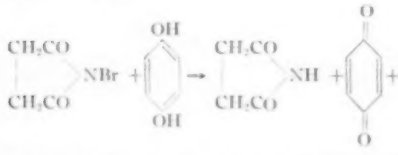
RESULTS

Interfering Substances.—The only interfering substance that is also oxidized by N-bromosuccinimide before iodine is liberated from potassium iodide includes sulfite present in photographic developers but it is eliminated in the form of insoluble barium sulfite. Metol has no influence on the titration process.

Experimental Error.—From the results given in Tables I to IV, it has been deduced that the error of the proposed method does not exceed ±2%.

DISCUSSION

N-Bromosuccinimide in aqueous medium readily oxidizes an aqueous solution of hydroquinone to benzoquinone, while N-bromosuccinimide is irreversibly reduced to succinimide with the formation of hydrogen bromide. The reaction proceeds quantitatively, in the presence of sodium bicarbonate, in equimolecular concentrations according to the equation:



Sodium bicarbonate eliminates hydrogen bromide as soon as it is formed and consequently the reaction proceeds forward to completion. The fact that hydroquinone is selectively oxidized by N-bromosuccinimide (19, 20) before iodine is liberated from potassium iodide, provides a reliable titrimetric method for the determination of hydroquinone.

N-Bromosuccinimide is an oxidizing agent and thus can liberate iodine from potassium iodide in aqueous medium, but it oxidizes hydroquinone preferentially. Until all the hydroquinone present in the solution is oxidized, no iodine is liberated from potassium iodide. The slightest excess of N-bromosuccinimide added, after all the hydroquinone content has been oxidized, will liberate iodine from potassium iodide, which is easily detected by the blue color developed with a few drops of starch solution added at the beginning of the titration process. The end point is definitely blue in the case of pure solutions but violet in the case of fats, oils, and photographic developers.

The proposed method is applicable even to such low concentration as 55 mcg. of hydroquinone. Only 0.01% N-bromosuccinimide is used in the titration process and a blank experiment is simultaneously carried out. The blank reading (0.09 ml. of 0.01% NBS) is subtracted from the titration result before calculation is made.

TABLE IV.—ESTIMATION OF HYDROQUINONE IN ETHER[a]

Volume of Ether, ml.	Hydroquinone Content, mg.	0.1% NBS Used, ml.	Hydroquinone Found, mg.	Error, %
1	10	16.20	10.01	0.10
10	10	16.30	10.07	0.70
8	8	13.10	8.10	1.25
6	6	9.80	6.06	1.00

[a] Dilution, 10×; 1 mg. per ml.

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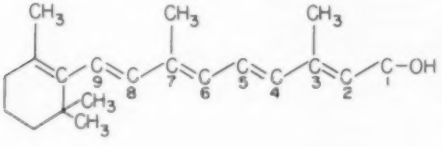
Isomerization of Vitamin A in Aqueous Multivitamin Drop Preparations*

By ROBERT W. LEHMAN, JOHN M. DIETERLE, WILLIAM T. FISHER, and STANLEY R. AMES

Seven samples of aqueous multivitamin drop formulations were prepared using three different samples of all-*trans*-vitamin A palmitate. These were assayed after three, six, nine, and twelve months' storage at 37°, and after nine, twelve, and fifteen months' storage at 25°. The rate of apparent deterioration of vitamin A was dependent on the assay method used, being least for the antimony trichloride blue-color assay, intermediate for the U. S. P. spectrophotometric assay, and greatest for the rat liver-storage bioassay. The vitamin A isomer composition was studied by reaction with maleic anhydride and by examination of infrared absorption spectra of purified vitamin A aldehydes made from the preparations. Results of this study indicate that during storage in the multivitamin drop preparations, all-*trans*-vitamin A isomerizes to a mixture containing not only all-*trans*- and 2-mono-*cis*- (neo-vitamin A), but also significant quantities of 6-mono-*cis*- and 2,6-di-*cis*-isomers, which have low biological activity.

VITAMIN A (see Fig. 1) has five conjugated double bonds, some of which can exist in either the *trans*- or *cis*- configuration. In 1939, Pauling (1) postulated that two of these double bonds, 2—3 and 6—7, were "stereochemically effective" and could exist readily in either the *trans*- or *cis*-configuration; and that other isomers were unlikely because they would be sterically hindered. These isomers have been prepared by Robeson, *et al.* (2), and their properties determined (2, 3, 4). Table I gives some of the analytical properties in terms of the "potencies" obtained by different assay procedures.



all-trans — 6-mono-cis
2-mono-cis (neo) — 2,6-di-cis

Fig. 1.—Isomers of vitamin A.

EXPERIMENTAL

In our present studies we prepared seven aqueous multivitamin drop preparations. They were made with commercial, all-*trans*-vitamin A palmitate, contained Tween 80 as the dispersing agent, and were adjusted to pH 5.3. All contained about 6,000 U. S. P. units of vitamin A, 1 mg. of thiamine, and 50 mg. of ascorbic acid in a "dose" of 0.6 cc. In addition, four of the preparations also contained 0.4 mg. riboflavin, 2 mg. calcium pantothenate, and 5 mg. nicotinamide per 0.6 cc. The preparations were divided into individual bottles and tightly capped under nitrogen for assay after storage for three, six, nine, and twelve months at 37°, and for nine, twelve, and fifteen months at room temperature (controlled at 25°).

The rate of apparent deterioration of vitamin A was measured by three different assay methods: the antimony trichloride blue-color procedure (4), the U. S. P. XV spectrophotometric procedure (5), and the rat slope-ratio liver-storage biological assay (6).

The recovery data obtained for the preparations stored at 37° are summarized in Fig. 2. Antimony trichloride blue-color determinations and U. S. P. spectrophotometric assays were obtained on practically all of the preparations at all storage periods. Bioassays were obtained on several of the preparations. Similar data at 25° are given in Fig. 3.

It is apparent that two different changes are taking place at the same time. The vitamin A is decomposing chemically; and this is the change of greatest magnitude. However, as decomposition takes place, there arises a systematic discrepancy between three different assay procedures. The apparent rate of decomposition is least by the antimony trichloride blue-color assay; it is intermediate by the U. S. P. XV assay; and it is greatest by the rat liver-storage bioassay.

One explanation for the divergence of the three assay procedures is that vitamin A isomerizes. Reference to Table I will illustrate how vitamin A isomers respond to the different assay methods. A mixture of the four isomers should be expected to have a lower "potency" by U. S. P. XV assay than by antimony trichloride blue-color, since all four isomers give the same colorimetric value while three of the isomers have lowered U. S. P. values. Likewise, the biological potency of a mixture of four isomers should be lower than that obtained by either of the physico-chemical assay procedures.

The per cent recoveries found by each assay procedure, as presented in Figs. 2 and 3, are averaged

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The expert technical assistance of H. A. Risley and W. J. Swanson of the D.P.I. Biochemical Research Department, M. H. Stern and W. P. Blum of the D.P.I. Organic Research Department, and H. W. Rawlings and G. H. Wait of the D.P.I. Manufacturing Control Laboratory greatly assisted the progress of this investigation.

TABLE I.—PROPERTIES OF GEOMETRIC ISOMERS OF VITAMIN A PALMITATE[a]

Isomer	Antimony Trichloride Blue-Color "Potency," u./Gm.	U. S. P. XV "Potency," u./Gm.	Relative U. S. P. XV "Potency," % of Blue-Color	Biological Potency, u./Gm.	Relative Biopotency, % of Blue-Color	Relative Biopotency, % of U. S. P. XV
All-trans	1,818,000	1,818,000	100	1,818,000	100	100
2-mono-*cis*	1,818,000	1,301,000	72	1,370,000	75	105
6-mono-*cis*	1,818,000	1,263,000	69	413,000	23	33
2,6-di-*cis*	1,818,000	1,320,000	73	413,000	23	31

[a] From data on vitamin A acetates published by Robeson, *et al.* (2), Ames, *et al.* (3), and Embree, *et al.* (4), calculated stoichiometrically for the palmitate ester, since none of the properties given is affected significantly by the ester form.

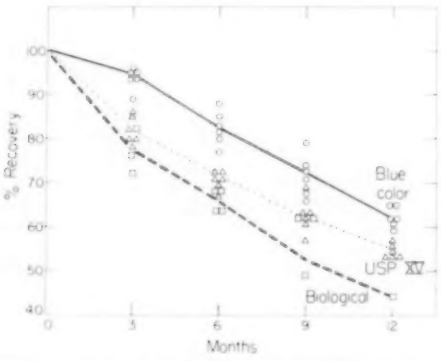

Fig. 2.—Vitamin A recovery in multivitamin drop preparations stored at 37°.

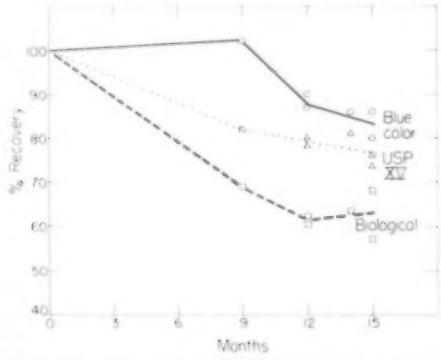

Fig. 3.—Vitamin A recovery in multivitamin drop preparations stored at 25°.

in Table II. In interpreting these data, we consider that chemical destruction of vitamin A is most accurately measured by the change in the blue-color assay; and that the ratios of U. S. P. XV potency to blue-color potency and of biopotency to blue-color potency are indicators of isomerization. Thus, the effect of isomerization, independent of chemical destruction, can be seen in Table III. Here the relative potencies are shown at each storage period. The U. S. P. XV and biological "potencies" are expressed as a percentage of the blue-color "potency," and the biopotency is expressed as a percentage of U. S. P. XV "potency." Results are shown both at 37 and 25° storage.

TABLE II.—% RECOVERY OF VITAMIN A IN AQUEOUS MULTIVITAMIN DISPERSIONS

	Months 3	6	9	12	15
37°					
Antimony Trichloride Blue-Color	94	82	72	62	..
U.S.P. XV	82	71	62	55	..
Biological	77	66	53	44	..
25°					
Antimony Trichloride Blue-Color	..	..	102	88	84
U. S. P. XV	..	..	82	79	77
Biological	..	..	69	62	63

TABLE III.—"RELATIVE POTENCY" OF VITAMIN A IN AQUEOUS DISPERSIONS

	Months 3	6	9	12	15
37°					
U. S. P. XV/Antimony Trichloride Blue-Color	86	85	86	88	..
Biological/U. S. P. XV	94	92	84	80	..
Biological/Antimony Trichloride Blue-Color	81	78	72	70	..
25°					
U. S. P. XV/Antimony Trichloride Blue-Color	..	..	83	89	92
Biological/U. S. P. XV	..	..	78	76	77
Biological/Antimony Trichloride Blue-Color	..	..	65	68	71

Upon storage, the relative biopotency drops within the first few months of storage and then levels off at a value near 70% of the antimony trichloride blue-color value. Likewise, the relative biopotency drops and levels off at about 80% of the U. S. P. XV value. It appears that most of the isomerization effect takes place during the first few months of storage.

Another indicator of isomerization is the reaction of vitamin A with maleic anhydride. This has been used (2, 4, 7) to determine the proportion of the total vitamin A present that has a *cis*-configuration at the 2-position. Expressed as "maleic value," its relationship to biopotency is the subject of a companion paper (8).

Table IV presents the averages of the maleic values obtained at 37° and at 25° for the different storage periods. Upon storage at 37°, the maleic value levels off at about 25%, while at room temperature it reaches 34%. It is still uncertain whether

TABLE IV.—MALEIC VALUE OF VITAMIN A IN AQUEOUS DISPERSIONS

	Months					
	0	3	6	9	12	15
37°	6	22	27	26	24	..
25°	6	..	..	27	30	34

or not the final equilibrium is the same at 25° as a 37°.

A third measure of isomerization depends on the infrared absorption curves of purified vitamin A aldehydes (9, 10, 11). Figure 4 shows the infrared absorption curves over a very narrow wavelength range for all-*trans*- and 6-mono-*cis*-vitamin A aldehydes. At 8.6 μ the all-*trans*-vitamin A has an absorption peak where the 6-mono-*cis*-isomer has an absorption minimum. The reverse occurs just below 8.75 μ. In the remainder of the infrared region, the absorption curves of these two isomers are nearly identical. The absorption curve for 2-mono-*cis*-vitamin A is similar to that of all-*trans* while the curve for 2,6-di-*cis*- is similar to that of the 6-mono-*cis*-isomer.

Two of the aqueous multivitamin drop preparations in the present study were examined by infrared absorption after storage for one year at room temperature. They were saponified, the unsaponifiable fractions oxidized to vitamin A aldehyde, and then purified for infrared analysis. Both were estimated (10) to contain 18% of their vitamin A in the 6-mono-*cis*- and 2,6-di-*cis*-forms. A third preparation was similarly examined after fifteen months' storage at room temperature. It was estimated to contain 21% combined 6-*cis*-isomers.

CONCLUSIONS

All-*trans*-vitamin A isomerizes on storage in aqueous multivitamin drop preparations. The resulting equilibrium mixture contains isomers of much lower biological potency than all-*trans*- and 2-mono-*cis*-vitamin A. The relationships between the different "potencies" found by rat liver-storage bioassay, by U. S. P. XV spectrophotometric assay, and by antimony trichloride blue-color assay indicate that the 6-mono-*cis*- and 2,6-di-*cis*-isomers are formed; their presence is confirmed by infrared absorption measurements. These effects are in addition to the chemical decomposition of vitamin A that takes place at the same time.

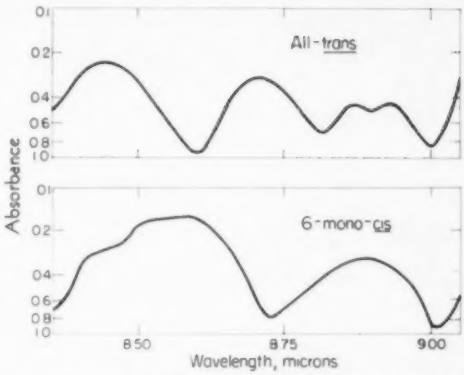

Fig. 4.—Infrared absorption curves, partial, for all-*trans*- and 6-mono-*cis*-vitamin A.

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Estimation of the Biological Potency of Isomerized Vitamin A Palmitate in Aqueous Multivitamin Dispersions from Maleic Values*

By STANLEY R. AMES, WILLIAM J. SWANSON, and ROBERT W. LEHMAN

The relative biopotencies on a blue-color basis and the maleic values of isomerized all-*trans*-vitamin A palmitate from stored aqueous multivitamin dispersions are related by the cubic regression equation: relative biopotency = 99.5 − 0.2(MV) − 0.051(MV)² + 0.000768(MV)³. Using this equation, the vitamin A biopotency of aqueous multivitamin dispersions can be computed from chemical data. This cubic equation relating the relative biopotency and maleic value was successfully applied to a number of mixtures of vitamin A isomers of both synthetic and natural origin. The presence of 6-*cis*-isomers of low biological potency in such mixtures of vitamin A isomers is indicated.

VITAMIN A PALMITATE, stored in aqueous multivitamin dispersions, has been reported by Lehman, *et al.* (1), to isomerize to a mixture of isomers. Not only was 2-mono-*cis*-(neo) vitamin A formed, but, in addition, substantial amounts of the 6-*cis*-isomers (6-mono-*cis*- and 2,6-di-*cis*-) were formed. The degree of isomerization can be readily determined by bioassay of the aqueous dispersion. However, there is real need for a rapid chemical procedure to estimate the biopotency of a mixture of vitamin A isomers. The data in this communication further substantiate the report of Lehman, *et al.* (1), that isomers of low biological potency (6-*cis*-isomers) are formed in aqueous multivitamin dispersions. Relative biopotency and maleic value are related by a cubic equation.

EXPERIMENTAL

Preparations Tested.—Some of the aqueous multivitamin dispersions were prepared in the laboratory as previously described (1). Others were samples of commercial products of known composition and length of storage. All samples had initially been prepared from all-*trans*-vitamin A palmitate.

Maleic Value Determination.—Maleic anhydride forms a condensation product of the Diels-Alder type with those vitamin A isomers in which both the 2—3 and 4—5 double bonds are *trans*. The use of this reagent for the assay of neovitamin A in mixtures with all-*trans*-vitamin A was described by Robeson and Baxter (2). The reaction of maleic anhydride with 6-mono-*cis*-vitamin A was described by Robeson, *et al.* (3). Details of the procedure have been previously described by Embree, *et al.* (4). Thus, the maleic value measures the amount of unreacted 2-mono-*cis*- and 2,6-di-*cis*-vitamin A. The maleic value is computed from the following equation:

$$\text{Maleic value } (\%) = \frac{R - R_1}{R_2 - R_1} \times 100$$

where R = recovery of vitamin A in the test sample. The constants R_1 and R_2 have been recently re-evaluated (5): for vitamin A esters $R_1 = 2.0$ and $R_2 = 93.4$; for vitamin A alcohol $R_1 = 1.1$ and $R_2 = 89.5$.

Bioassay Procedure.—The vitamin A biopotencies of aqueous multivitamin dispersions were determined by the slope-ratio liver-storage procedure of Ames and Harris (6). In this procedure, rats are supplemented with 1,000 to 2,000 units of vitamin A during a one- to three-day period. The livers are removed forty-eight hours after the final dose and analyzed for vitamin A by the procedure of Ames, Risley, and Harris (7). Some aqueous dispersions were administered by quantitative oral injection into the rat's stomach and compared with U. S. P. vitamin A reference solution (8) similarly administered. In most cases, the aqueous dispersions were saponified and an oil solution of the nonsaponifiable matter administered orally by dropper and compared with nonsaponifiable fraction from the U. S. P. vitamin A reference solution similarly administered.

In this report

$$\text{Relative biopotency } (\%) = \frac{\text{Biopotency}}{\text{Chemical potency by antimony trichloride (blue-color) determination}^1} \times 100$$

since the determination of vitamin A with antimony trichloride is the best measure of total vitamin A, irrespective of isomeric structure (4). The relative biopotencies for the four vitamin A isomers are: all-*trans*-, 100%; 2-mono-*cis*-, 75%; 6-mono-*cis*-, 23%; and 2,6-di-*cis*-, 23%, as previously reported by Ames, *et al.* (9).

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The expert technical assistance of H. A. Risley, J. M. Dieterle, and W. T. Fisher of the Research Laboratories, and H. W. Rawlings and G. H. Wait of the Manufacturing Control Laboratory greatly assisted the progress of this investigation.

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[1] The U. S. P. vitamin A reference solution was used as the standard for the antimony trichloride (blue-color) determination (4) of vitamin A.

RESULTS

A series of aqueous multivitamin dispersions of all-*trans*-vitamin A palmitate were stored for varying lengths of time. Maleic values and relative biopotencies on a blue-color basis were determined. The results of fifty-three analyses, as plotted in Fig. 1, indicated that the relative biopotency varied inversely with the maleic value. The linear regression of relative biopotency on maleic value was computed, but on inspection failed to fit the observed data for maleic values less than ten. However, the cubic regression of relative biopotency on maleic value showed the required curvature at low maleic values, and fit the data more closely than either the linear or quadratic regression. Coefficients for the quadratic and cubic terms are statistically significant, as shown in Table I. The equation for the cubic regression plotted in Fig. 1 is

$$\text{Relative biopotency} = 99.5 - 0.2(\text{MV}) - 0.051(\text{MV})^2 + 0.000768\,(\text{MV})^3$$

with standard deviation of the calculated relative biopotency minus observed relative biopotency equal to 3.55.

TABLE I.—SIGNIFICANCE OF THE CUBIC REGRESSION

$$y = a + bx + cx^2 + dx^3$$

Term	Coefficient	σ (coefficient)	t (coefficient)
Constant (a)	99.5	0.89	111.0[a]
Linear (b)	0.2	0.33	0.491
Quadratic (c)	0.051	0.022	2.30[b]
Cubic (d)	0.000768	0.000379	2.03[b]

$df = 48$. $p = 0.05$. $t = 2.01$.

[a] Highly significant.
[b] Significant.

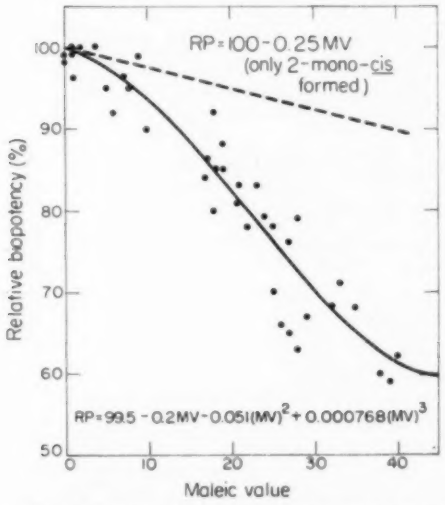

Fig. 1.—Relative biopotency (RP) *vs.* maleic value (MV) for all-*trans*-vitamin A isomerized in aqueous multivitamin dispersions.

The relative biopotency of a mixture of all-*trans*- and 2-mono-*cis*-vitamin A can readily be computed from maleic values by the linear equation plotted in Fig. 1. The bioassay results found with all-*trans*-vitamin A palmitate stored in aqueous multivitamin dispersions can not be explained by the isomerization of all-*trans*-vitamin A only to the 2-mono-*cis*-isomer. These data further substantiate the conclusions of Lehman, *et al.* (1), that in an aqueous dispersion all-*trans*-vitamin A isomerizes to a mixture of isomers including the 6-mono-*cis*- and 2,6-di-*cis*-isomers, which have low biological potency.

Several previous attempts to relate the relative biopotency with various chemical or spectral properties of the vitamin A isomers met with limited success. The use of least squares statistics to relate the relative biopotency and maleic value is empirical, but offers the best practical solution yet developed. The linear, quadratic, and cubic regressions of relative biopotency on maleic value were compared. The quadratic coefficient in the quadratic regression was not statistically significant, and this equation was therefore rejected. The cubic regression equation is preferred to the linear form because of both a better fit to the observed data at low maleic values and a lesser overall standard deviation of calculated minus observed relative biopotency. The cubic form of the regression equation suggests that initially, all-*trans*-vitamin A palmitate isomerizes more rapidly to the 2-mono-*cis*-isomer which has 75% of the biopotency of all-*trans*-vitamin A than to the 6-*cis*-isomers which have only 23% of the biopotency of all-*trans*-vitamin A.

This isomerization of all-*trans*-vitamin A palmitate in aqueous multivitamin dispersions appears to reach an equilibrium. Even after prolonged storage, the relative biopotency does not drop below 60–70% nor does the maleic value increase to values greater than 30–40%. Such an equilibrium mixture has been previously described by Ames, *et al.* (10), for vitamin A isolated from rat livers, and corresponds to a mixture of the four isomers in which the ratio of *trans*- to *cis*-isomer about each of the two double bonds, 2—3 and 6—7, is 2 to 1. Chemical isomerization of 2-mono-*cis*-vitamin A esters results in an isomer mixture with similar properties (3). Thus, a similar mixture of vitamin A isomers can be formed by isomerizing either all-*trans*- or 2-mono-*cis*-vitamin A.

APPLICATION TO OTHER ISOMER MIXTURES

The cubic regression equation described above has shown excellent results in predicting relative biopotencies from observed maleic values for vitamin A palmitate stored in aqueous multivitamin dispersions. Application of this equation to other mixtures of vitamin A isomers was tested. Preisomerized vitamin A palmitates were obtained by chemically isomerizing the all-*trans*-isomer by various procedures (2, 11). Twenty-seven such preparations were analyzed for both relative biopotency and maleic value. The results, plotted in Fig. 2, show excellent agreement with the cubic regression equation described above. Those preisomerized vitamin A palmitates with relative biopotencies and maleic values corresponding to an equilibrium

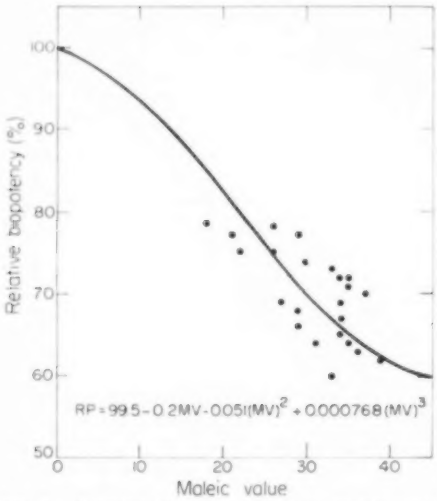

Fig. 2.—Relative biopotency (RP) *vs.* maleic value (MV) for preisomerized vitamin A preparations.

mixture of isomers exhibited no significant isomerization on storage in aqueous multivitamin dispersions (12).

Several naturally-occurring vitamin A oils have been found to contain substantial quantities of 6-*cis*-isomers (13). Such mixtures of vitamin A isomers have been isolated from fish-liver oils and lipid extracts of both rat and human livers. The relative biopotencies and maleic values for these preparations are correlated by the above cubic regression equation. These data suggest that the cubic regression of relative biopotency on maleic value may have general application to mixtures of vitamin A isomers from both synthetic and natural sources.

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The Isolation of β-Allocryptopine from *Argemone squarrosa* subsp. *squarrosa**

By T. O. SOINE and R. E. WILLETTE†

An examination of the alkaloidal content of *Argemone squarrosa* subsp. *squarrosa* has yielded approximately 1 per cent of β-allocryptopine as the major alkaloid, if not the only one. The alkaloid has been identified by conventional derivatives as well as by comparison of its infrared spectrum with that of its physical isomeride, α-allocryptopine. Pharmacological testing has indicated that the alkaloid is relatively inactive except for its known ability to suppress auricular fibrillation.

STUDIES in these laboratories previously have involved the investigations of *Argemone hispida* (1, 2) and *A. munita* subsp. *rotundata* (Rydb.) (3) with specific reference to their alkaloidal content. These studies led to the isolation of three alkaloids provisionally named argemonine, norargemonine, and rotundine. Unpublished studies since that time have led to the belief that these alkaloids are representatives of the isoquinoline group and are specifically of the aporphine type. It was of interest, therefore, to examine another species, namely *Argemone squarrosa* subsp. *squarrosa* to see whether it too contained alkaloids derived from the aporphine type of isoquinoline alkaloids.

A preliminary assay indicated a rather favorable alkaloidal content. Ethanolic extractions made in a Soxhlet and a Lloyd apparatus, however, afforded a lower alkaloidal yield than that

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possible according to the assay. A much more satisfactory yield was obtained by utilizing modified Prollius fluid in a percolator of our own design. In all cases, the total yield was comprised almost entirely of a single alkaloid which was conclusively identified as β-allocryptopine, by means of physical properties, derivatives, and infrared spectra.

With respect to infrared comparisons, it was noted that there were minor differences in oil mull preparations between the dimorphic forms. These differences, due to crystal structure, were eliminated by comparing the two samples in solution, in which case the dimorphism was no longer operative.

Allocryptopine has been isolated previously from various sources (4), and from the *Argemone* genus has been reported from *A. mexicana* (5). The alkaloid is dimorphic, the lower melting α-form, being known also as β-homochelidonine, and the higher melting β-form, being known also as α-fagarine and γ-homochelidonine.

PHARMACOLOGY

The alkaloid has received considerable attention pharmacologically (6, 7). It has been claimed to be five times as active as quinidine in suppressing auricular fibrillation (6, 8) and has found some use in South America for this purpose (9). Unfortunately, it has a tendency to initiate ventricular fibrillation which imposes serious limitations on its use.

Prior to our identification, pharmacological tests[1] indicated that the base was relatively inactive. With respect to the central nervous system the drug failed to affect the qualitative behavior of the dog at 20 mg./Kg. or the spontaneous activity of the rat at a dose of 81 mg./Kg. At a dose of 300 mg./Kg. in the mouse, 1/2 died and excitement, tremor, and convulsions were evident. The base was also inactive with respect to the blood pressure, diuretic action, anti-inflammatory action (iritis), ankle edema, and the tracheal chain test (bronchial relaxation).

EXPERIMENTAL

Material.—The dried, whole supraterranean plant of *Argemone squarrosa* subsp. *squarrosa*,[2] collected in southeastern Colorado in the Las Animas area, was ground to a coarse powder in preparation for extraction.

Extraction.—The ground plant (15.45 and 15.48 Gm.) was assayed according to the nux vomica assay procedure (10) and gave 1.39% and 1.64%, respectively, total alkaloidal content. The ground plant (400 Gm.) was then extracted for one hundred and sixteen hours with 95% ethanol in a Soxhlet apparatus until negative to Mayer's T. S. The extract was concentrated to a syrup *in vacuo*, dissolved in methylene chloride, extracted with 10% hydrochloric acid, basified and extracted with methylene chloride, which upon concentration yielded 3.5 Gm. (0.87%) of total alkaloid. When the ground drug (6 Kg.) was extracted in a Lloyd apparatus and the extract processed in like manner, only 25 Gm. (0.42%) was obtained.

Inasmuch as the assay utilized an ether:chloroform:ammonia solvent, the ground plant (3 Kg.) was extracted by shaking it with 5 L. of modified Prollius fluid (ether:chloroform:ethanol:concentrated ammonium hydroxide, 250:55:25:10) in a 5-gallon bottle. It was allowed to stand overnight, the mouth provided with a suitable filter, the bottle inverted, air pressure applied to the bottom, and the contents allowed to percolate. This was repeated five times before a negative test was obtained with Mayer's T. S. This extract was concentrated to a syrup on a steam bath with the aid of a stream of air. It was dissolved in Skellysolve B and thoroughly mixed with 10% sulfuric acid, evaporating the organic solvent with a stream of air after it was allowed to rise to the top. This undisturbed mass was frozen and the acid-insoluble material separated from the top of the frozen acid solution which was very clear upon melting. The acid-insoluble residue was extracted twice more in this manner until negative to Mayer's T. S. This method proved far more satisfactory than extraction in a separatory funnel, in which case emulsions formed with considerable ease. The acid extract was alkalinized with concentrated ammonium hydroxide and, upon standing overnight, large masses of crystals had formed, which were filtered off, yielding 20 Gm. (0.67%) of β-allocryptopine. The filtrate was extracted with ten 250-ml. portions of methylene chloride, which upon concentration yielded a further 11 Gm. (0.37%) of alkaloid, from which 10 Gm. of β-allocryptopine was separated by crystallization, giving a total of over 1% of β-allocryptopine from the plant. The residues are being investigated for further alkaloids.

Identification of β-Allocryptopine.—The isolated alkaloid responded to all the usual color tests for the allocryptopines (11) such as a deep reddish-violet color with concentrated sulfuric acid and a positive methylenedioxy test. The base was recrystallized several times from 95% ethanol, dried at 100° *in vacuo* overnight, the long white needles melting at 169–171°[3] [(lit. m. p. 170–171° (4c, 11)]. A mixed melting point with an authentic sample of α-allocryptopine (m. p. 162°), graciously supplied by R. H. F. Manske, gave depression to 166°. When this melt was allowed to cool and recrystallize, it melted at 170°. All efforts to convert either sample back to the α-form failed.

Anal.[4]—Calcd. for $C_{21}H_{23}NO_5$: C, 68.28; H, 6.28; N, 3.79. Found: C, 68.30; H, 6.57; N, 3.75.

The infrared absorption spectra of the base (Fig. 1*a*), the sample of α-allocryptopine supplied by Manske and β-homochelidonine[5] (Fig. 1*b*) were determined using a Perkin and Elmer spectrophotometer,

[1] The authors acknowledge and appreciate the kind cooperation of G. D. Searle and Co., Chicago, Ill., in the pharmacological testing.

[2] The authors are indebted to Dr. Gerald B. Ownbey, Botany Department, University of Minnesota, for the identification of the plant material. The nomenclature employed in this paper is in accord with that in Ownbey, G. B., *Mem. Torrey Botan. Club*, **21**, (1959). A herbarium specimen has also been deposited in the archives of the Botany Department University of Minnesota.

[3] All melting points were determined with a Kofler Micro-melting point apparatus and are corrected.

[4] All analyses were carried out by the Microanalytical Laboratory, School of Chemistry, University of Minnesota, Minn.

[5] Obtained from L. Light & Co. Ltd., Colnbrook, England.

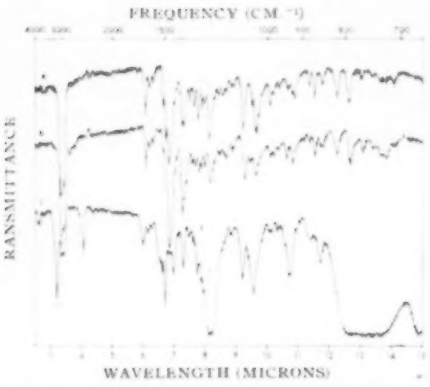

Fig. 1.—Infrared spectra of: (*a*), β-allocryptopine, Nujol mull; (*b*), α-allocryptopine (Manske) or β-homochelidonine (Light), Nujol mulls; (*c*), β-allocryptopine or β-homochelidonine in chloroformic solution.

Model 137. They showed some minor differences in oil mull preparations, but gave identical spectra (Fig. 1*c*) in chloroformic solution.

Hydrochloride.—This salt was prepared by extracting a dilute hydrochloric acid solution of the base with chloroform, which upon concentration gave very fine needles of the hydrochloride. Recrystallized from ethanol, it began to turn yellow at 170° and decomposed at 195° [lit. m. p. 190–192° (decompn.) (9) and m. p. 175° (11)]. Upon drying at 100° *in vacuo* overnight, it melted at 189–190°. It contains a molecule of solvent of crystallization (9).

Anal.—Calcd. for $C_{21}H_{23}NO_5 \cdot HCl \cdot C_2H_5OH$: C, 61.10; H, 6.64. Found: C, 60.99; H, 6.59.

Methiodide.—This derivative was prepared in the usual manner. Recrystallized from ethanol, the fine, white needles melted at 190–205° (decompn.) [lit. m. p. 205–206° (9)]. Upon drying at 100° *in vacuo* overnight, they melted at 204–205°.

Anal.—Calcd. for $C_{21}H_{23}NO_5 \cdot CH_3I$: C, 51.60; H, 5.08. Found: C, 51.88; H, 5.42.

Aurichloride.—This salt was prepared by precipitating the base with 0.2 *N* auric chloride T. S. It crystallized very slowly from ethanol giving characteristic warty, dark red crystals, melting at 187–187.5° [lit. B. $HAuCl_4$, m. p. 187–192° (9)].

Picrate.—This derivative was prepared in the usual manner and recrystallized from anhydrous ethanol, m. p. 208.5–209.5° [lit. m. p. 208–209° (4c)].

Anal.—Calcd. for $C_{21}H_{23}NO_5 \cdot C_6H_3N_3O_7$: C, 54.19; H, 4.44. Found: C, 54.18; H, 4.38.

SUMMARY

1. β-Allocryptopine has been isolated from *Argemone squarrosa* subsp. *squarrosa* in approximately 1 per cent yield.

2. The alkaloid has been identified by means of derivatives and comparison of its infrared spectrum with those of known samples of its physical isomeride, α-allocryptopine.

3. Some pharmacological data, in addition to that already in the literature, have been reported.

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The Application of Alternating Current Polarography in the Determination of Phenobarbital in Dosage Forms*

By N. G. LORDI, E. M. COHEN, and B. L. TAYLOR

The technique of alternating current polarography has been applied to phenobarbital, using a relatively simple modification of the Sargent model XXI polarograph. Distinctive analytically useful waves were obtained in pH 8 borate buffer containing 1 *M* potassium nitrate. Phenobarbital was determined in both liquid and solid dosage forms with an accuracy of 2 per cent by dilution of the sample and subsequent comparison of the wave scanned in buffer to that of a standard. Results indicated that the barbiturate could be determined directly in the presence of a wide variety of drugs without the necessity for isolation from the dosage form.

PRACTICAL METHODS for the determination of phenobarbital in its dosage forms have been reported by many workers. These may be conveniently divided into three groups, the first being gravimetric methods which involve isolation of the barbiturate from the dosage form and its subsequent weighing, an example of which is the present official U. S. P. method. The second group includes nonaqueous titrations (1, 2) using a variety of reagents and solvents which generally require prior extraction of the barbiturate from its dosage form. More recently, Vincent and Blake (3) effected the necessary separation of barbiturates from dosage forms with ion exchange resins prior to titration. The third group includes those methods which involve the direct titration of phenobarbital with a metal reagent which reacts with the barbiturate forming a weakly ionized or insoluble salt. Bodin (4) titrated phenobarbital potentiometrically in elixirs, parenteral solutions, capsules, and tablets with silver nitrate. Kalvoda and Zyka (5) employed mercuric nitrate in an amperometric technique using the dropping mercury electrode to determine phenobarbital in tablets and other solid mixtures. Cohen (6) has recently successfully titrated phenobarbital in elixirs with mercuric acetate, using either potentiometric or amperometric methods of end point detection.

In recent years the technique of alternating current polarography has come under close scrutiny as a means of extending the analytical usefulness of the polarographic method (7, 8). In this technique, a small sinusoidal alternating voltage is superposed on the direct potential normally applied to the dropping mercury electrode in conventional polarography. The alternating component of the current produced is measured and recorded as a function of the applied direct potential. The resulting graphs are referred to as a. c. polarograms. The most significant feature of these plots is observed when substances which are reversibly reduced are studied. A maximum alternating current (summit current) is exhibited at the direct potential (summit potential) which corresponds to within ±50 millivolts of the half-wave potential of the conventional polarogram. A. c. polarograms thus appear, at least in form, to be the first derivative of the d. c. polarogram.

Among the advantages a. c. polarography has over d. c. polarography is that oxygen need not be expelled from the solution to be analyzed, since substances which are irreversibly reduced and not adsorbed on the surface of the electrode do not exhibit a. c. polarograms. Thus, this technique may be used as a test for reversibility in redox processes. Other advantages include increased sensitivity and ease of separation of waves which occur at potentials close to one another.

In addition to substances which undergo reversible reduction or oxidation, solutions of surface-active materials produce wave-shaped current-voltage curves under a. c. conditions which have their origin in adsorption processes occurring at the electrode surface. Breyer and Hacobian (9) have termed this type of electrode process "tensammetry." In their words (10), tensammetric waves are a consequence of " . . . adsorption-desorption equilibrium at the electrode-solution interface without electron transfer." Breyer and Hacobian (11) have also recently applied tensammetry to the estimation of tungstate ion.

Experiments with phenobarbital, using the technique of alternating current polarography, revealed that it exhibited distinctive waves.

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Presented to the Scientific Section, A. PH. A., Cincinnati meeting, August 1959.

The purpose of this report is to describe the properties of these waves and to indicate an approach to their analytical utilization in pharmaceuticals.

EXPERIMENTAL

Apparatus.—The method of converting the Sargent model XXI polarograph to record a. c. polarograms, as originally described by Miller (12), was modified. The recording section of the Sargent polarograph is essentially a potentiometer in which a d. c. signal is converted into an a. c. signal which is in turn amplified and balanced against a standard reference signal. For this work the converter was removed from the amplifier and replaced by a six-prong plug with pins 3 and 4 shorted. This changed the recording section of the instrument from a d. c. measuring device into a high gain a. c. measuring device. The additional circuitry required is diagrammed in Fig. 1.

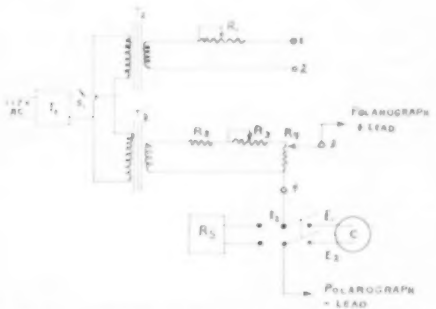

Fig. 1.—Electrical circuitry required for operation of Sargent model XXI polarograph as an a. c. polarograph.

C, polarographic cell; E_1, saturated calomel electrode; E_2, dropping mercury electrode; R_1, 5000 ohm potentiometer; R_2, 200 ohm, 1/2 watt resistor; R_3, 100 ohm potentiometer; R_4, 10 ohm potentiometer; R_5, decade resistance box; S_1, SPST switch; S_2, DPDT switch; T_1, constant votage transformer, 115 volts a. c.; T_2, filament transformer; primary, 115 volts a. c.; T_3, secondary, 2.5 volts a. c.

The output of one of the filament transformers (terminals 1 and 2 on the diagram) was connected across the terminals normally occupied by the 1½ volt working cell in the recorder. These terminals must be connected so that the recorder will read zero with the cell leads open and be driven upscale when the cell leads are shorted. This functioned to introduce an alternating voltage 180 degrees out of phase with the signal to be measured. Provision was made for substitution of a decade resistor in place of the polarographic cell for purposes of calibration. In addition, a 500-microfarad, 25-volt, electrolytic capacitor was fixed in position in the polarographic section between the movable arm of the slide wire and the fixed arm of the sensitivity selector. This capacitor was necessary to shunt the a. c. signal around the slide wire to prevent changing the resistance to a. c. as the resistance of the polarograph slide wire was changed. The presence of this capacitor did not affect the operation of the instrument as a d. c. polarograph.

All controls functioned as when the polarograph was operated as a d. c. device except the damping switch, which was, of necessity, kept in the off position. No attempt was made to calibrate the scale of the recorder in terms of absolute current. Rather the current scale used was expressed in arbitrary units obtained by multiplying the number of recorder chart[1] scale divisions by the sensitivity setting of the instrument. In this work, the voltage across R_4 was adjusted by means of R_3 to a value of 100 millivolts r. m. s. The calibration point was selected to give optimum response at a sensitivity setting of 0.1 on the instrument. When R_4 was adjusted to give an output of 30 millivolts r. m. s., R_1 was set so that, when a resistance of 1,000 ohms was substituted for the cell at a sensitivity setting of 0.1, the pen was deflected to exactly 15.0 on the recorder scale. After each run this setting was readjusted to allow for the small amount of drift which was observed to occur.

A conventional H-type cell (13) was employed with a 1.5 M potassium nitrate agar plug to minimize diffusion of chloride into the cell from the reference electrode (a saturated calomel electrode[2]). The characteristics of the dropping mercury electrode capillary (length: 9 cm.) were such that, when operated under a pressure of 39 cm. of mercury, the drop time at 25° was 2.57 seconds in 1 M KNO_3 at 0 applied potential and m was 3.26 mg. $sec.^{-1}$

No exact theoretical interpretation could be made of the a. c. polarograms obtained with the apparatus described (8), since no provision was made for maintaining a constant a. c. voltage across the mercury-solution interface and no attempt was made to correct for the series resistance of the cell. However, since the intent behind this work was to develop an empirical analytical method for phenobarbital, the additional instrumentation required was not deemed essential to the achievement of this purpose.

The Beckman model G pH meter was employed for all measurements.

Reagents.—Phenobarbital U. S. P. (m. p. 176–177.5° uncorrected), was recrystallized twice from alcohol-water. Phenobarbital sodium U. S. P., was dried for several hours at 110° prior to use. The buffer used in this work consisted of 2.5 M potassium nitrate and 0.125 M boric acid to which 0.012 M sodium hydroxide was added. A 2.5-fold dilution of this buffer had a pH of 7.9. Reagent grade chemicals were employed unless otherwise stated.

Preliminary Experiments.—Initial efforts were directed towards determining those conditions under which the optimum phenobarbital wave could be elicited. Parameters evaluated were scanning rate, pH, ionic strength, drop time, magnitude of applied a. c. voltage, and temperature. Most experiments were carried out at a room temperature of 25 ± 0.5°, using 30 millivolts r. m. s. applied a. c. Scanning rates of 1.23 and 0.62 millivolts per second were used. When quantitative results were desired the lower scanning rate was used, since true values of

[1] Beckman chart No. 6353-N.
[2] Beckman model 1170-71.

summit current could only be obtained at low scanning rates or when the instrument was operated manually (14). Furthermore, the maxima of the current oscillations were used as the basis for reference in measuring current.

The waves of both phenobarbital and sodium phenobarbital were first evaluated in 1 *M* potassium nitrate. Typical waves obtained are shown in Fig. 2. It was noted that at low concentrations the waves of both the acid and salt forms of the barbiturate were nearly identical, while an eightfold increase in concentration caused significant changes both in wave shape and current magnitude. This was to be expected, since 10^{-4} *M* sodium phenobarbital is, for all practical purposes, completely hydrolyzed in the potassium nitrate media.

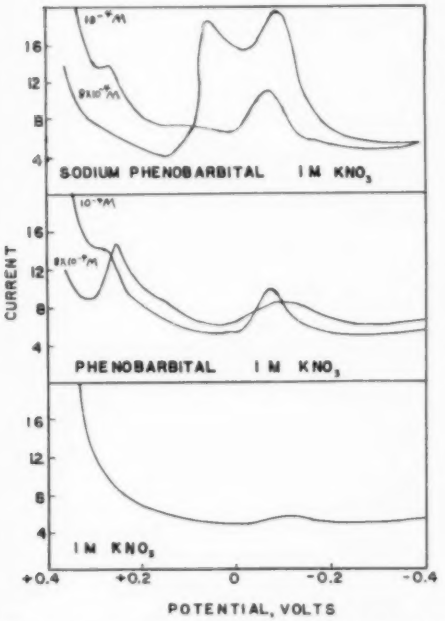

Fig. 2.—A. c. polarograms of phenobarbital and its sodium salt in 1 *M* KNO_3.

The pH of a solution, 1 *M* in potassium nitrate and initially containing 0.05 *M* boric acid and 10^{-4} *M* phenobarbital to which 0.05 *M* sodium hydroxide had been added, was varied by the addition of increments of 2 *M* perchloric acid. The waves were scanned after the addition of each increment of acid. The necessity for pH control can be seen upon examination of Fig. 3. Below pH 7 the phenobarbital wave had disappeared. Optimum sensitivity, i. e., maximal summit current, was attained in the vicinity of pH 8. An increase in pH above this point tended to reduce both the sensitivity and resolution (degree of separation from the residual current) of the wave.

Reduction in the total ionic content of the supporting electrolyte also diminished both the sensitivity and resolution of the wave, as well as increased the magnitude of the residual or capacitance current which was a result of the charging of the mercury drop by the supporting electrolyte. However, sensitivity was again reduced at ionic strengths greater than 1.

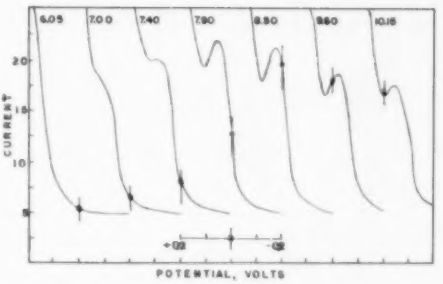

Fig. 3.—pH dependence of phenobarbital wave in borate buffer: 1 *M* KNO_3 and 10^{-4} *M* phenobarbital.

The current was a function of the applied a. c. voltage, an increase in voltage tending to increase the current. However, increases in voltage to values greater than 30 mv. r. m. s. diminished the resolution of the waves. Reduction in voltage to a minimum of 10 mv. r. m. s. tended to improve resolution, especially at low concentrations. In the vicinity of room temperature the summit current was observed to fluctuate less than 0.5% per degree, tending to increase with temperature. The dropping rate was also observed to affect summit current, a change in drop time from 2.0 to 4.8 seconds increasing the current about 20%.

Further experiments indicated that the decomposition products of phenobarbital, obtained by heating a solution of the sodium salt in the presence of a tenfold excess of sodium hydroxide, produced no waves. This established the validity of using a. c. polarography as a means of assaying for phenobarbital.

Effect of Phenobarbital Concentration.—Typical phenobarbital waves recorded in pH 8 borate buffer are illustrated in Fig. 4. In order to establish the concentration dependence of the wave form, the summit current corrected for the residual current of the blank, i. e., the peak current, was plotted as a function of concentration. The resultant curve (Fig. 5) was nonlinear, an analytically useful standard curve being obtained only at concentrations lower than 10^{-4} *M*. No significant changes in summit current were observed when the concentration of barbiturate was increased to 0.01 *M*.

Procedure for Assay of Liquid Dosage Forms. Elixirs and Parenteral Solutions.—A suitable aliquot of solution, containing about 20 mg. of phenobarbital, was diluted to volume in a 100-ml. volumetric flask with distilled water. One- or two-milliliter aliquots of this dilution were added to 10 ml. of buffer concentrate in a 25-ml. volumetric flask and brought to volume with distilled water. Each sample was scanned at a rate of 0.62 mv. $sec.^{-1}$ and the peak currents computed from the resulting waves. The concentration of barbiturate in the dosage form was calculated from an appropriate standard curve.

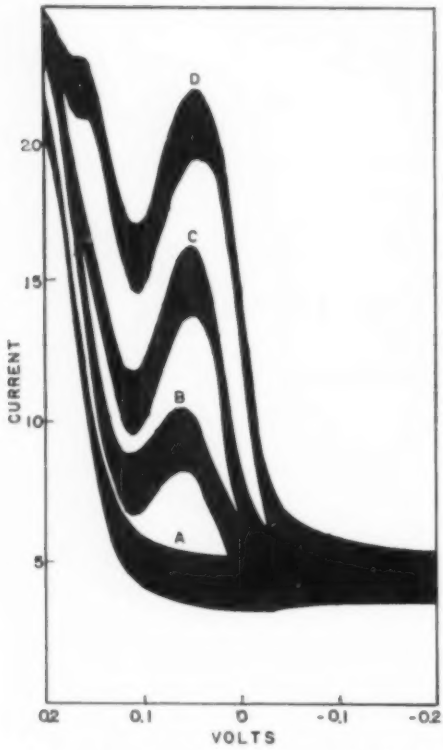

Fig. 4.—A. c. polarograms of phenobarbital in borate buffer: pH 7.9 and 1 *M* KNO_3. Phenobarbital concentration: *A*, none; *B*, 2×10^{-4} *M*; *C*, 5×10^{-4} *M*; and *D*, 10^{-3} *M*.

Procedure for Assay of Solid Dosage Forms: Powders, Capsules, and Tablets.—Accurately weighed, uniformly mixed samples containing approximately 20 mg. of phenobarbital were added to 10 ml. of alcohol U. S. P. in a 100-ml. volumetric flask. The mixture was agitated to extract the barbiturate from the solid before diluting to volume with distilled water. The addition of alcohol was omitted if the barbiturate was present as a water-soluble salt. Where necessary, the resultant mixture was centrifuged to remove insoluble matter before 1- or 2-ml. aliquots were taken for analysis in the manner already described.

The results obtained for the recovery of phenobarbital from some typical dosage forms are summarized in Table I.

Effect of Foreign Substances on the Phenobarbital Wave.—A study was made of the possible interference of other drugs which are commonly formulated with phenobarbital and certain other substances on the wave form. The phenobarbital wave was scanned in the buffer at a concentration of 10^{-4} *M* in the presence of a ten-fold excess of the drug. The results indicated that the method was not applicable to the determination of the barbiturate in the presence of caffeine, diphenylhydantoin, aminophylline, theobromine sodium salicylate, thiamine hydrochloride, bromide and iodide. These substances tended to distort the wave and/or change the character of the residual current. Aspirin, phenacetin, sodium salicylate, atropine sulfate, ephedrine sulfate, and chloride did not interfere. All the halides produced distinctive waves in the absence of phenobarbital, the summit potential of only chloride being significantly more positive (0.1 volts) than that of phenobarbital.

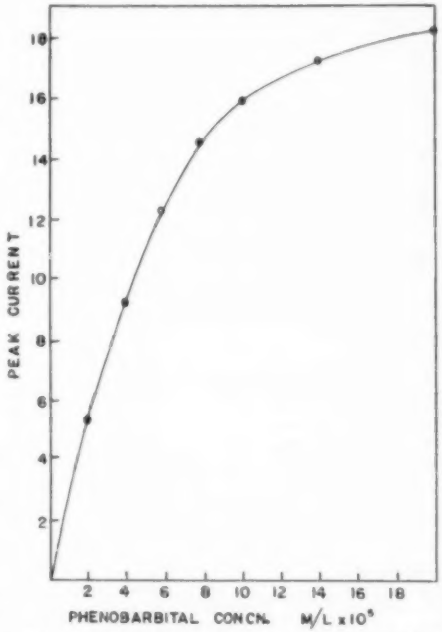

Fig. 5.—Concentration-peak current standard curve of phenobarbital in borate buffer: pH 7.9 and 1 *M* KNO_3.

TABLE I.—ANALYSIS OF PHENOBARBITAL IN DOSAGE FORMS

Dosage Form	No. Samples	Recovery, %
Phenobarbital elixir, U. S. P. XV	8	101.0 ± 0.6
Phenobarbital sodium injection[a]	6	99.5 ± 1.4
Tablet mixture[b]	9	98.7 ± 0.8
Lactose dilution[c]	6	99.3 ± 1.7

[a] Propylene glycol solution containing 20 mg. of phenobarbital sodium per ml.
[b] Tablet formulation described by Bodin (4) containing 15 mg. of phenobarbital in each 200 mg.
[c] 1:10 trituration of phenobarbital in lactose.

Concentrations of alcohol and propylene glycol less than 1% did not alter the peak current of phenobarbital significantly, while higher concentrations tended to reduce sensitivity as well as increase residual current. Polyethylene glycol 400 interfered in concentrations as low as 0.5%, tending to increase the residual current greatly. Strong surface active materials, e. g., sodium stearate and methylcellulose, depressed the peak current to a significant extent.

DISCUSSION

The experimental data indicated that the successful application of alternating current polarography to the determination of phenobarbital in dosage forms depended, in part, upon the high dilution factors employed which minimized the effect of diluents, excipients, flavors, colors, and other foreign ingredients on the analytical phenobarbital wave. A case in point was phenobarbital elixir. The usual aliquot chosen for analysis was 5 ml., the sample employed to record the wave being a 250- to 500-fold dilution of the elixir. At these dilutions the components of the elixir base had no effect on the character of the wave. The particular tablet mixture studied contained, in addition to the barbiturate, magnesium stearate, acacia, starch, sucrose, and lactose, none of which affected the wave in the amounts normally present in tablets.

The degree of accuracy attainable, about 2%, was limited by the nature of the standard curve. The error involved in interpolation from the curve was further compounded by the necessity of using high dilution factors.

The principal advantages of the proposed method were its rapidity and the elimination of the need for isolation of the barbiturate from its dosage form. In many cases the method could be extended to mixtures containing interfering substances if blank corrections were made for the residual current and the calibration curve prepared in the presence of the interfering substances.

Unlike the d. c. polarographic diffusion current, the alternating current measured in a. c. polarography is not dependent on concentration gradients near the electrode, but is a function of the concentration of active species at the electrode surface. As a consequence, when the electrode surface has become saturated, maximum peak current will have been attained. The form of the peak current-concentration curve will approximate that of an adsorption isotherm (Fig. 5).

The a. c. polarographic behavior of phenobarbital differed in several important respects from the behavior attributed by Breyer and Hacobian (9) to tensammetric processes. These investigators observed that the effect of an increase in temperature was to decrease peak current in the case of substances which exhibited positive temperature coefficients of solubility, while the reverse was true in the case of those substances which exhibited negative solubility temperature coefficients (e. g. methylcellulose). They also observed that weak acids and bases (e. g., phenol and pyridine) were effective in producing tensammetric waves only in the undissociated state. In contrast to these observations it was found that phenobarbital showed greater peak current at elevated temperatures and was more effective in the salt form. Furthermore, phenobarbital showed no wave on the negative side of the electrocapillary zero, a characteristic of tensammetric processes, and produced well-defined waves at very low concentrations (e. g., 10^{-6} *M*).

Phenobarbital appeared to correspond more closely in behavior to the halides which exhibit a. c. polarographic waves referred to by Breyer and Hacobian (10) as "transition" waves. Unlike the true tensammetric wave, transition waves are a result of two processes: a polarographic step, involving interaction of the active species with the electrode to produce a surface-active product, and a tensammetric process, involving an adsorption-desorption equilibrium at the electrode surface. Thus, the a. c. waves of the halides are the result of reaction with mercury to form mercurous halide (i. e., the electrode reaction responsible for the existence of anodic d. c. polarographic waves) which is adsorbed on the mercury drop.

Phenobarbital is not polarographically reduced but does show an anodic wave (15). The a. c. polarographic behavior of phenobarbital, as indicated by these observations, appears to be a consequence of a reaction of phenobarbital with mercurous ion formed by a polarographic electrode reaction, the resultant mercurous phenobarbital being adsorbed at the mercury-solution interface.

Preliminary experiments were run with other barbiturates, i. e., pentobarbital, amobarbital, and secobarbital. All exhibited a. c. polarograms similar in form to those of phenobarbital. It is to be expected that the analytical technique described should be generally applicable to all barbiturates. A more extensive study of the a. c. polarography of the barbiturates will be the subject of a later paper.

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Study of the Stability of Secobarbital Sodium Solutions I*

Determination of Solubility of Secobarbital as a Function of Solvent and Hydrogen Ion Concentration

By J. H. UDANI and J. AUTIAN

The solubility of secobarbital in water at various temperatures was determined and from this information a plot was prepared, log S *vs.* $1/T$. The linear relationship indicated the adherence of the curve to van't Hoff's equation. From the slope, the heat of solution was calculated and found to be 4358.0 cal./deg./mole. Solubility studies were also undertaken to determine the quantity of secobarbital which would dissolve in various binary solvent systems in which water was always the second solvent. The order of solubility increased from glycerin–propylene glycol–PEG 400–alcohol. Glycerin had very little solubilizing activity for secobarbital even in volume concentrations up to 50 per cent. In a three-component solvent system composed of alcohol: glycerin : water, the glycerin produced a synergistic effect on the dissolution reaction. Finally, the influence of pH on solubility of secobarbital in water alone and in several mixed solvents was ascertained, and the results plotted as solubility curves. It was also demonstrated that alcohol, propylene glycol, glycerin, and PEG 400 suppressed ionization of secobarbital in aqueous solutions. All of the solvents except PEG 400 showed a linear relationship in the solvent concentration studied.

STUDIES on aqueous solutions of the barbiturates indicate that pH and temperature will have an appreciable effect upon the stability of the drug. These studies further elucidate that as the hydrogen ion concentration and temperature are increased, the degradation proceeds rapidly. In order to circumvent the use of the salt of the particular barbituric acid, the acid form of the drug is employed with the addition of alcohol, propylene glycol, polyethylene glycols, or other agents to achieve dissolution of the relatively insoluble barbituric acid in water. The acidic pH thus insures greater product stability by decreasing the hydrolysis reaction known to occur in the alkaline pH range (1–3).

In this study, secobarbital sodium was selected as the barbiturate to investigate. Before undertaking a vigorous investigation of secobarbital stability, however, it became necessary first to accumulate data on the solubility of the drug as a function of solvent and pH in order to design the stability experiments properly. The information reported in this paper describes the solubility phase of the study.

EXPERIMENTAL

Apparatus and Supplies.—Leeds & Northrup pH meter (Cat. 7664); shaking apparatus in constant temperature bath; secobarbital and secobarbital sodium U. S. P.; absolute alcohol U. S. P.; propylene glycol U. S. P.; glycerin U. S. P.; polyethylene glycol U. S. P.; absolute methyl alcohol, R. G.; chloroform, R. G.; potassium hydroxide, R. G.; thymol blue solution (0.5% w/v in methyl alcohol).

Assay of Secobarbital.—To determine the secobarbital in the various samples quantitatively, a nonaqueous titration method was used. The assay employed chloroform as the solvent and a standard (0.1 N) solution of potassium hydroxide in absolute methyl alcohol as the titrant, using thymol blue solution as the indicator. The exact procedure for the assay is given by Chatten (4). This method was compared with an ultraviolet spectrophotometric assay suggested by Mattson (5) and found to agree within experimental errors.

Solubility as Function of Temperature.—An excess quantity of secobarbital was added to 50-ml. Erlenmeyer flasks containing 30 ml. of freshly distilled water, previously heated to the desired temperature. Three such solutions were prepared for each temperature studied. The flasks were then stoppered, placed into a constant temperature bath at the desired temperature, and agitated for a period of twenty-four hours. The solution from each flask was then quickly filtered and an aliquot (approximately 5 ml.) transferred to a weighing bottle of known weight. After the bottle containing the solution was weighed, the liquid was poured into a 125-ml. separatory funnel. The bottle was rinsed with three separate portions (5 ml.) of distilled water and the combined rinse water added to the separatory funnel. Fractions of chloroform (25 ml.) were then used to extract the secobarbital for assay. In all instances, the volume of chloroform was concentrated to 50 ml. before proceeding with the assay. In this experiment, solubility was determined at 20, 25, 30, 35, 40, 45, and 50° ± 0.2°. The results were based on the average of three samples for each temperature.

The data were plotted as log S *versus* $1/T$ and found to be linear in the range studied (Fig. 1). The

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The authors wish to express their sincere appreciation to Dr. Ewald Rohrmann, Eli Lilly and Co., for the generous supply of secobarbital and secobarbital sodium.

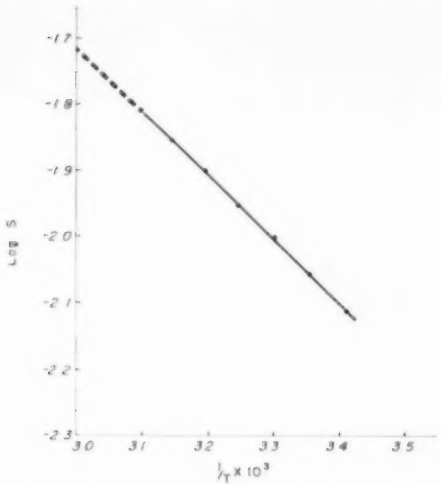

Fig. 1.—Solubility of secobarbital in water as function of absolute temperature.

linear relationship indicates its conformity to van't Hoff's equation

$$\log S = \frac{-\Delta H}{2.303\,RT}\,1 + \text{constant} \qquad \text{(Eq. 1)}$$

where S is the solubility in moles per 1,000 Gm. of solvent at the absolute temperature T, R is the gas constant, and ΔH the heat of solution in calories per degree per mole. From the slope $-\Delta H/2.303R$ the heat of solution (ΔH) was calculated and found to be 4358.0 cal./deg./mole.

Solubility as Function of Solvent.—Excess quantities of secobarbital were added to various concentrations of alcohol, propylene glycol, glycerin, and polyethylene glycol 400, water making up the remainder of the solvent. A 30-ml. sample for each concentration and solvent system was prepared, placed into a constant temperature bath at 30° ± 0.2°, and agitated for twenty-four hours. The secobarbital was extracted as described before for each sample and assayed for drug content. The results were based upon the average of three samples for each concentration and solvent system. Figure 2 presents the data in graphical form.

The solubility of secobarbital in a three-component solvent system was determined and the data are plotted in Fig. 3 as per cent solubility *versus* glycerin added to alcohol base. In each instance, the remaining quantity of solvent was water.

Solubility as Function of pH.—The desired quantity of secobarbital sodium (from a theoretical solubility curve) was weighed on a prescription balance and placed into a 50-ml. beaker containing 25 ml. of distilled water. Hydrochloric acid (1.0 N) was then added, dropwise, to adjust the pH to the requisite value, and the volume of the solution was brought up to 30 ml. with distilled water. This solution was then transferred to a 50-ml. Erlenmeyer flask, stoppered, and placed into a constant temperature bath at 30° ± 0.2°. A number of such solutions were prepared, each at a differ-

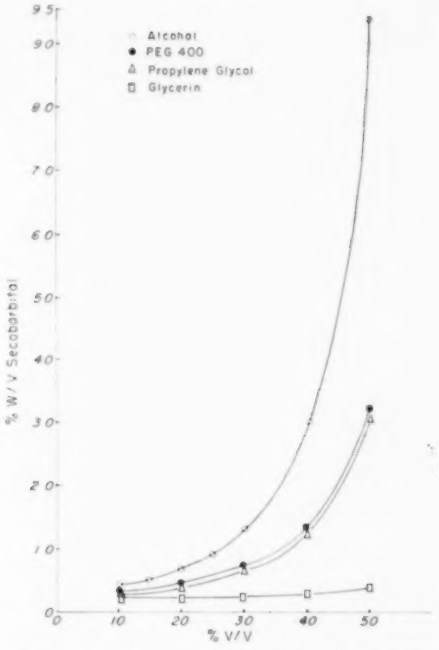

Fig. 2.—Solubility of secobarbital as function of solvent system and concentration.

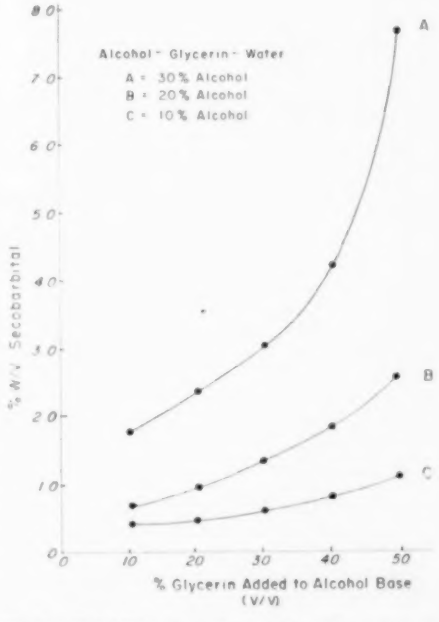

Fig. 3.—Solubility of secobarbital in a three-component solvent system

ent pH, and the flasks agitated for a period of twenty-four hours. In all instances, at each pH value, an excess of secobarbital was maintained.

At the end of the agitation period, the flasks were removed and the pH of the supernatant liquid determined. Each sample (supernatant liquid) was then acidified with 1.0 *N* hydrochloric acid to convert the salt into its acid form and extracted with chloroform in a manner described previously. The samples were then assayed for their drug content. For each pH value, duplicate samples were run. Table I includes the experimental results of solubility at the final pH. A solubility curve was drawn from the data in the table and is included in Fig. 4 (in water). In the same figure appears a theoretical solubility curve calculated from an equation developed by Higuchi (6)

$$S = (HA)(1 + K_a/H^+) \qquad \text{(Eq. 2)}$$

where S is the total solubility of secobarbital, expressed in moles per liter, (HA) the acid solubility in the same units, K_a[1] the dissociation constant of secobarbital in water at 30°, and H^+ the hydrogen ion concentration. The calculated solubility values are included in Table I.

TABLE I.—SOLUBILITY OF SECOBARBITAL IN WATER AS A FUNCTION OF pH

Experimental		Theoretical[a]	
pH	Gm./100 ml.	pH	Gm./100 ml.
2.20	0.223	2.00	0.214
4.20	0.223	4.00	0.214
5.10	0.223	5.00	0.214
6.30	0.231	6.00	0.217
6.70	0.248	7.00	0.241
7.42	0.297	7.50	0.299
7.75	0.446	8.00	0.484
8.30	1.0750	8.50	1.069
8.50	1.653	9.00	2.916
8.55	1.943	9.40	7.002
8.90	4.591		

[a] Calculated from Eq. 2, using 1.26×10^{-3} as K_a and 9.0 $\times 10^{-4}$ as (HA) concentration.

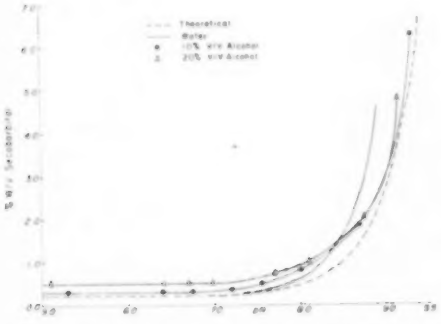

Fig. 4.—Solubility of secobarbital in alcohol-water systems as a function of pH.

Solubility as Function of Solvent and pH.—A series of solubility studies were conducted as described in the previous section except the binary

[1] K_a is equal to 1.26×10^{-3}.

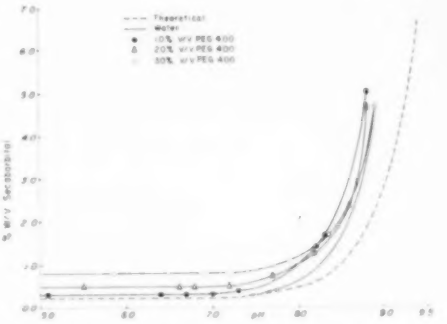

Fig. 5.—Solubility of secobarbital in PEG 400-water systems as a function of pH.

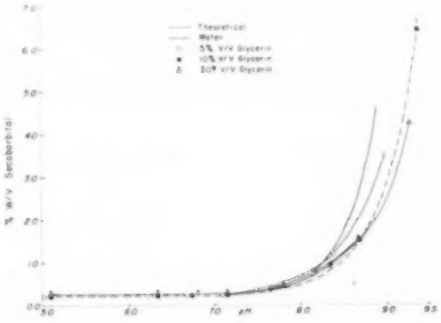

Fig. 6.—Solubility of secobarbital in glycerin-water systems as a function of pH.

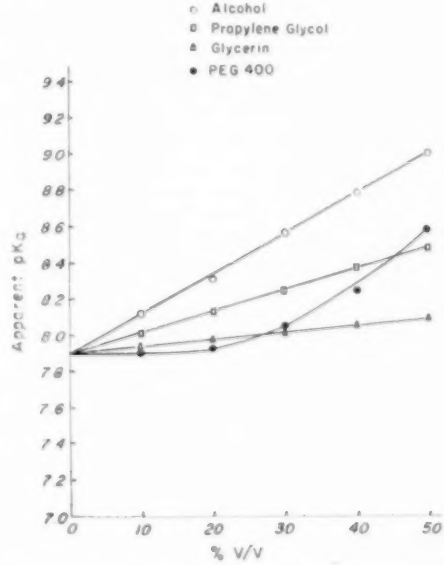

Fig. 7.—Effect of solvent on pKa of secobarbital.

solvent systems were composed of various concentrations of either alcohol, glycerin, or polyethylene glycol 400 with water. The data obtained from these studies were plotted as shown in Figs. 4 to 6.

Effect of Solvent upon Apparent pKa Value.—In order to determine the effect of the dissociation of secobarbital in various concentrations of alcohol, propylene glycol, glycerin, and polyethylene glycol 400, solutions were prepared containing a 1:1 salt to acid concentration. Each solution contained 0.26028 Gm. of secobarbital sodium to which was added exactly 5.00 ml. of a 0.1 *N* hydrochloric acid. The solutions were then adjusted to the proper per cent (v/v) of solvent system, placed into a water bath at 30° for four hours, and the pH of each solution was then determined. Since in a 1:1 buffer system the pH is equal to the pKa, a plot was drawn in Fig. 7 relating pKa to the various solvent concentrations.

RESULTS AND DISCUSSION

Temperature Effect.—The linear relationship shown in Fig. 1 indicates that the solubility is related to the reciprocal of absolute temperature as predicted by van't Hoff's equation. Since the heat of solution (ΔH) is known, the solubility at any other temperature may be calculated by the use of another form of Eq. 1

$$\log \frac{S_2}{S_1} = \frac{\Delta H(T_2 - T_1)}{2.303\ R\ (T_2T_1)} \qquad \text{(Eq. 3)}$$

where S_1 and S_2 are the solubility in moles per 1,000 Gm. of solvent at absolute temperatures T_1 and T_2, respectively.

The twenty-four-hour agitation period was found to be adequate to insure that equilibrium had been reached in the solution. Previous studies substantiated the absence of hydrolysis at the higher temperatures within the twenty-four-hour period for even those solutions having an alkaline pH.

Solvent Effect.—The phase diagram in Fig. 2 may conveniently be employed to find the exact solubility of secobarbital in concentrations (10–50% v/v) of alcohol, polyethylene glycol 400, propylene glycol, or glycerin at 30°. It will be noted that alcohol, in the higher concentration range, imparts the greatest degree of solubility. This is not too surprising, since, in general, a solvent system having a low dielectric constant should have greater dissolution activity to a relatively nonpolar compound such as secobarbital (undissociated). From the binary solvent systems included in the figure, alcohol-water has the lowest dielectric constant.

Polyethylene glycol 400 and propylene glycol dissolve approximately the same concentration of secobarbital in the 10–50% v/v concentration range of solvent. However, as may be seen in the figure, these two solvent systems have approximately one-half the solubilizing effect at 40% v/v concentration and one-third the effect at the 50% v/v concentration range, in comparison to alcohol.

In the solvent range studied, glycerin proved to be a rather poor solvent since even up to 50% v/v concentration, it had little ability to increase the solubility. Krause and Cross (7) found this to be true in their study on the solubility of phenobarbital.

Glycerin appears to have a synergistic solubilizing action upon alcohol-water systems. If Fig. 3 is studied closely and then compared to Fig. 2, it will be noted that in all instances glycerin increases the solubilizing effect over the binary system, alcohol-water. For example, to dissolve 5% w/v of secobarbital in 100 ml. of a solvent system, a 45% v/v alcohol-water system must be used, but this same quantity of secobarbital may be dissolved in an alcohol-glycerin-water system having volume concentrations of 30%, 45%, and 25%, respectively. Glycerin-water systems up to 50% barely solubilize 0.4% w/v of secobarbital.

pH Effect.—Even though the theoretical solubility curve for secobarbital was calculated using Eq. (2), the classical Henderson-Hasselbalch equation based upon the law of mass action may have been used.

$$\text{pH} = \text{pKa} + \log \frac{(\text{Salt})}{(\text{Acid})} \qquad \text{(Eq. 4)}$$

However, this equation will express only the salt concentration, and at relatively high hydrogen ion concentration will not be useful since the concentration of salt to acid will be insignificant. In the case of secobarbital, the results by Eqs. 2 or 4 are practically identical above pH values of 7.0.

As may be seen by examining Fig. 4, the theoretical and experimental curves are in good agreement at the low pH values but diverge at the higher values. This finding is quite common when apparent dissociation constants and molar concentrations are used, rather than thermodynamic dissociation constants and activities. The equations, however, may be used to calculate approximate solubilities at a certain pH. Often this type of solubility calculation is very helpful to the product development group in formulation or in predicting incompatibilities.

Solvent and pH Effect.—Alcohol had a tendency to increase the solubility, as could be predicted, at the lower pH values and to decrease the solubility at the higher pH values. Polyethylene glycol 400 increased the solubility at all pH levels compared to the solubility of secobarbital in water. The excellent solvent and stabilizing action of the glycols for the barbiturates may be attested to by their inclusions in commercial barbiturate solutions. Glycerin decreased the solubility of secobarbital in the higher pH range. Unfortunately, time did not permit a more intensive investigation with higher concentrations of solvent as well as other solvent systems.

Solvent Effect upon pKa.—It is well known that less polar solvents than water will suppress ionization of weak organic acids. This may be seen by referring to Fig. 7 where apparent pKa values are plotted against volume concentrations of the solvents. All lines intersect at a pKa value of 7.9, which is the pKa value of secobarbital in water. The unlinear relationship with polyethylene glycol 400 is indicative that a molecular intereaction between the solute and solvent has probably occurred.

SUMMARY AND CONCLUSION

1. The solubility of secobarbital in water at various temperatures was determined and from this information the heat of solution was calculated. The heat of solution was found to be 4358.0 cal./deg./mole.

2. Binary solvent systems in which water was always the second solvent were found to increase the solubility in the order: glycerin–propylene glycol–PEG 400–alcohol.

3. Glycerin imparted a synergistic solubilizing activity in a three-component solvent system (alcohol-glycerin-water) upon the secobarbital.

4. Solubility curves as per cent w/v secobarbital *versus* pH were plotted from experimental data in water and in several mixed solvent systems.

5. The apparent pKa for secobarbital in various concentrations of alcohol, propylene glycol, glycerin, and polyethylene glycol 400 were also determined and found to be linear in the range studied, except for polyethylene glycol 400.

6. Stability studies of secobarbital solutions are now in progress and will be presented in future papers.

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Study of the Stability of Secobarbital Sodium Solutions II*

Separation and Identification of Degradation Products of Secobarbital Sodium

By A. J. KAPADIA and J. AUTIAN

A study was conducted to separate and identify degradation products of secobarbital sodium in an aqueous medium. Thermally degraded secobarbital sodium solutions were employed in the study. Extraction and paper chromatographic techniques were used to separate three degradation products of secobarbital sodium. Infrared analysis and chromatographic techniques identified the products as (*a*) mono ureide of 1-methylbutyl allyl malonic acid, (*b*) 1-methylbutyl allyl acetyl urea, and (*c*) urea. A fourth compound, 1-methylbutyl allyl acetic acid, was formed in the degradation but could not be isolated. The steps in the degradation of secobarbital sodium parallel those reported by Rotondaro for phenobarbital sodium.

SINCE THE TURN of the century, many reports have been published upon the accelerated decomposition of the various barbiturates in alkaline solutions. One of the first references to the decomposition of a barbituric acid derivative was found in a German patent issued in 1903 (1). The patent included information that diethylacetyl urea was formed on heating barbital. Husa and Jatul (2) reviewed the literature and presented a summary of the pathways of the decomposition of barbituric acid derivatives in an alkaline medium. Decomposition products of phenobarbital sodium have been isolated and identified by Rotondaro (3). He identified the products as phenylethylacetyl urea, phenylethyl acetic acid, and urea. By paper chromatographic techniques Kapadia, Goyan, and Autian (4) affected a separation of the previously mentioned decomposition products of phenobarbital sodium.

Even though secobarbital has been in common medical use for some time, little published information is available concerning the products of degradation. This particular investigation was initiated to elucidate the decomposition products occurring in alkaline solutions of secobarbital and to compare the degradation steps to those of phenobarbital.

EXPERIMENTAL

Supplies.—Whatman filter paper No. 1; secobarbital sodium U. S. P.; secobarbital; Urea, A. R.; *n*-hexyl alcohol, A. R.; ammonium hy-

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Grateful acknowledgment is given to Dr. Harold Boaz, Eli Lilly and Co., Indianapolis, Ind., for the infrared and the titration data and their interpretation and to Mr. Ralph Huges for the microanalysis.
The authors wish to express their sincere appreciation to Eli Lilly and Co., for the generous supply of secobarbital.
Presented to the Scientific Section, A. PH. A., Cincinnati meeting, August 1959.

droxide (28%) A. R.; borate buffer (pH 10) which consists of 43.9 ml. sodium hydroxide (0.1 *N*), 50.0 ml. boric acid (0.1 *M*), and purified water to make 100.0 ml.; potassium permanganate solution (0.02 *N*); *p*-dimethylaminobenzaldehyde solution (2% in 1.2 *N* HCl).

Extraction Procedure.—Secobarbital sodium solution (6% w/v) was prepared using purified water as the solvent and placed into ampuls. The ampuls were sealed and thermally degraded by storing at 80° for four days. After the storage period, several of the ampuls were broken and the solution filtered to recover the existing precipitate. The precipitate was washed and recrystallized several times from warm water. This compound was found to be neutral and had a melting point of 120°.[1]

The infrared spectrum of this compound is essentially identical with that of *n*-butyl allyl acetyl urea at the same concentration in chloroform except in the methyl and methylene CH stretching and bending regions where it differs slightly, but in the expected way for the disappearance of one CH_2 and the addition of one —CH_3. As a reaction product of secobarbital this compound can be none other than 1-methylbutyl allyl acetyl urea (Fig. 1).

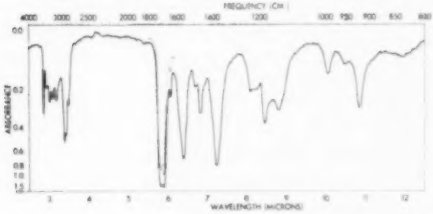

Fig. 1.—Infrared spectrum of 1-methylbutyl allyl acetyl urea in chloroform.

Approximately 20 ml. of the filtrate was placed into a 125-ml. separatory funnel and acidified with a few drops of dilute hydrochloric acid and extracted with 25 ml. of chloroform. This chloroform fraction was then transferred to another separatory funnel containing 25 ml. of a 0.1% sodium bicarbonate solution. The funnel was agitated slowly for several minutes and the organic phase removed. The remaining aqueous fraction was acidified with dilute hydrochloric acid and extracted with four portions of chloroform (20 ml.). These chloroform fractions were then pooled and the solvent evaporated. In order to obtain sufficient quantities of this compound, the above procedure was repeated a number of times, combining the resulting precipitates. Finally, the combined precipitate was recrystallized several times from methyl alcohol. The compound obtained was acidic in nature with a melting point of 140°.[1] The infrared spectrum, nitrogen analysis, and microtitration indicated that the compound was the mono ureide of 1-methylbutyl allyl malonic acid (Fig. 2).

Paper Chromatographic Separation.—An ascending paper chromatographic technique was employed for the separation of the several decomposition

[1] Uncorrected temperature.

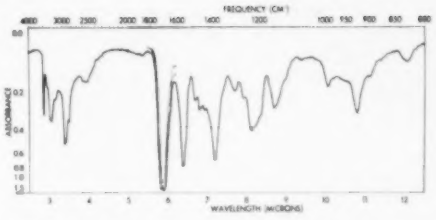

Fig. 2.—Infrared spectrum of the mono ureide of 1-methylbutyl allyl malonic acid in chloroform

products occurring in thermally degraded secobarbital sodium solution.

A two phase solvent system was employed in this study, consisting of the combination of the following solvents: *n*-hexyl alcohol, 200 ml.; borate buffer (pH 10), 25 ml.; ammonium hydroxide (28%), 12.5 ml.; purified water, 12.5 ml.

The above solvents were mixed in a separatory funnel for five minutes and then allowed to stand for two hours at room temperature, at which time the organic phase was removed. This phase was then used as the mobile solvent in the development of the chromatogram. The filter paper was sprayed with borate buffer (pH 10) and dried before use.

Aqueous solutions of secobarbital sodium and urea as well as chloroform solutions of 1-methylbutyl allyl urea and the mono ureide of 1-methylbutyl allyl malonic acid were spotted on the filter paper at equal intervals and at a starting point one and one-half inches from the bottom of the paper. These spots appear on the chromatogram (Fig. 3) at points A–D. At point E, spots were placed of the compounds mentioned above, superimposed upon each other. A spot of the aqueous fraction of the decomposed secobarbital sodium solution was placed at point F and a spot of the chloroform fraction at point G. A mixture of the aqueous and the chloroform (F and G) fractions were spotted at point H.

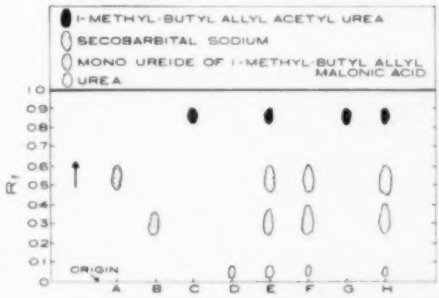

Fig. 3.—Separation of the decomposition products of secobarbital sodium by paper chromatography.

The paper was then placed into a battery jar containing a beaker of *n*-hexyl alcohol saturated with borate buffer-ammonium hydroxide-water solution. After an equilibration period, the mobile solvent system was added to the bottom of the jar. The chromatogram remained in the jar for a period

of eighteen hours, at which time it was removed and dried.

Three of the compounds (secobarbital, 1-methylbutyl allyl malonic acid, and 1-methylbutyl allyl acetyl urea) were viewed by spraying the chromatogram with 0.02 *N* potassium permanganate solution. For these compounds, a yellow spot on a red background was seen. The formation of these colors is indicative of compounds containing allyl groups. A bright yellow color developed for urea when the chromatogram was sprayed with *p*-dimethylaminobenzaldehyde solution.

The chromatographic technique made it possible to separate a number of the decomposition products from secobarbital sodium (Fig. 3). R_f values of the various compounds are listed in Table I.

TABLE I.—R_f VALUES FOR DECOMPOSITION PRODUCTS OF SECOBARBITAL SODIUM

Name of Compound	R_f Value[a]
Secobarbital sodium	0.58
Mono ureide of 1-methylbutyl allyl malonic acid	0.26
1-Methylbutyl allyl acetyl urea	0.88
Urea	0.09

[a] These values are the average of four determinations.

DISCUSSION

It was assumed that alkaline degradation of secobarbital paralleled the steps reported by Rotondaro for phenobarbital in alkaline solutions. If this is the case then the more important degradation steps can be depicted as shown in Fig. 4.

Fig. 4.—Degradation steps of secobarbital in alkaline solution.

A hydrolytic cleavage occurs in the secobarbital(I) molecule producing the acidic compound(II), mono ureide of 1-methylbutyl allyl malonic acid, which decarboxylates to yield the neutral compound, 1-methylbutyl allyl acetyl urea (III). Further hydrolysis of this compound, gives 1-methylbutyl allyl acetic acid (IV) and urea (V).

It is interesting to note that neither Rotondaro (3) nor Kapadia (4) reported the isolation of the mono ureide of phenylethyl malonic acid (analogous to II) as a decomposition product of phenobarbital, even though Rotondaro includes the compound as a decomposition product. In this particular study, compound IV could not be isolated by the techniques employed.

Since very little information was available as to actual decomposition products in secobarbital sodium solutions, it was first decided to isolate the several compounds by extraction methods. The thermal degradation accelerated the decomposition of the drug and produced a white precipitate as the first visible sign of decomposition. This precipitate was separated from the solution, purified, and subsequently identified as the neutral compound (III).

Acidulation of the alkaline solution and extraction with chloroform removed acidic and neutral compounds from the solution. Treatment of the chloroform fraction with bicarbonate solution separated the acidic decomposition product from secobarbital and any neutral product remaining. The pH of the bicarbonate solution was not sufficiently basic to convert the acid, secobarbital, to its salt form; thus, secobarbital remained in the chloroform phase. Finally, to isolate the acidic decomposition product from the aqueous phase from any neutral products, the solution was acidulated and the compound extracted with chloroform. On recovery of the compound and purification, the acidic compound was identified as the mono ureide of 1-methylbutyl allyl malonic acid (II).

With two of the decomposition products isolated and identified the paper chromatographic technique was developed to assist in rapid separation of the several degradation products occurring in secobarbital sodium solutions. This technique satisfactorily separated all the compounds shown in Fig. 4, but did not isolate compound IV. A number of methods of isolation were utilized to separate the compound but all proved fruitless.

SUMMARY

1. A study was undertaken to isolate and identify the several decomposition products occurring in alkaline solutions of secobarbital.

2. By extraction procedure and infrared analysis it was possible to separate and identify one acidic compound (mono ureide of 1-methylbutyl allyl malonic acid) and one neutral compound (1-methylbutyl allyl acetyl urea).

3. Paper chromatographic techniques made it possible to separate the mono ureide of 1-methylbutyl allyl malonic acid, 1-methylbutyl allyl acetyl urea, and urea from decomposed secobarbital sodium solutions.

4. The results of the study indicate that the alkaline degradation of secobarbital solutions parallel those steps reported for phenobarbital.

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The Hydrolysis of Hydrocortisone Phosphate in Essentially Neutral Solutions*

By ARNOLD D. MARCUS

The degradation of hydrocortisone phosphate in essentially neutral solutions appears to involve hydrolysis as the only significant degradative pathway. The reaction is first order with respect to the steroid ester and seems linearly dependent upon the hydrogen ion concentration. The reaction is apparently an unusual 0.44 order with respect to the catalyst. The activation energy for the overall reaction has been found to be 17.0 ± 0.5 Kcal./mole. The "frequency factor" of the Arrhenius equation has been calculated and an equation expressing the effect of both temperature and pH on the rate of the reaction is presented.

DURING THE FORMULATION of aqueous solutions of hydrocortisone phosphate it became apparent that the steroid ester was subject to both hydrolytic and oxidative degradation. Unfortunately, while oxidation could be minimized by adjusting the solutions to acid pH levels, the rate of hydrolysis increased markedly as the pH was lowered. Since, however, oxidation could be successfully controlled by the use of suitable antoxidants, the main problem concerned determination of the apparent minimum pH at which hydrolysis would proceed sufficiently slowly to permit adequate shelf lives for the formulated solutions.

Preliminary evidence, obtained by following the rate of formation of inorganic phosphate and/or the appearance of free hydrocortisone alcohol, indicated that the minimum suitable pH would be between 6 and 8. The rate of hydrolysis of hydrocortisone phosphate in this pH range was, therefore, studied with particular attention to the effect of temperature on the rate of reaction.

While the present study was by no means a comprehensive kinetic evaluation of the stability of hydrocortisone phosphate, it did provide the desired information in minimal time. It served, thereby, as an example of how chemical kinetic concepts and techniques permit most efficient use of accelerated stability testing programs.

GENERAL DISCUSSION

Hydrocortisone phosphate is a dibasic acid, pK_1 *ca.* 2 and $pK_2 = 6.2$. In the pH range 6 to 8, therefore, the ester could be presumed to be present both as the mono-anion and as the di-anion. Because of the negatively charged substrates, it appeared most likely that hydrolysis would be hydrogen ion-catalyzed. While hydroxyl ion-catalyzed hydrolysis could not be ruled out, the likelihood of electrostatic repulsion of a negatively charged catalyst appeared to minimize this latter possibility. Indeed, other studies of the hydrolysis of structurally analogous phosphate ester (1, 3–5), showed precisely the behavior assumed above.

If it can be assumed that both anionic forms of hydrocortisone phosphate are subject to hydrogen ion catalyzed hydrolysis and that both reactions are first order with respect to each of the reactants, the general rate equation can be given as:

$$\frac{d(HCPO_4)}{dt} = k'_{H^+}(H^+)(HCPO_4^-) + k''_{H^+}(H^+)(HCPO_4^{=}) \quad \text{(Eq. 1)}$$

where $(HCPO_4)$ is the total concentration of hydrocortisone phosphate, $(HCPO_4^-)$ the concentration of the mono-anion and $(HCPO_4^{=})$ the concentration of the di-anion. k'_{H^+} and k''_{H^+} are the specific rate constants characteristic of the indivdual reactions.

Since the two anionic forms of the substrates should hydrolyze at different rates and inasmuch as the relative concentration of each species changes with variations in the pH of the solution, it appeared unlikely that there would be any simple, linear, relationship between the rate of reaction and the pH of the system.

EXPERIMENTAL

Reagents.—Methylene chloride, Merck reagent, Porter-Silber reagent (2), 0.2 *M* phosphate buffers, pH 6–7.5.

Method of Degradation.—Exactly 2 Gm. of hydrocortisone phosphate, calculated as the free acid, was dissolved in a sufficient amount of each of the buffer solutions to yield exactly 200 ml. of solution. The solutions were sparged with nitrogen, subdivided into 5-ml. ampuls and sealed under nitrogen. The sealed ampuls were stored at 5° prior to starting the kinetic run.

For the kinetic runs, the ampuls were placed in a constant temperature bath set variously at 100, 91, 80, and 70 ± 0.1°. When thermal equilibrium was attained one ampul representing each pH was removed, immediately chilled in an ice-water mixture, and stored at 5° until the contents were analyzed. These initial samples were arbitrarily designated the "zero-hour" samples. Other ampuls were removed at suitable intervals, chilled, and stored in the same manner.

Analytical Procedure.—Exactly 2 ml. of the solution in each ampul (at room temperature) was placed

* Received August 21, 1959, from the Merck, Sharp & Dohme Research Laboratories, West Point, Pa.
Presented to Scientific Section, A. PH. A., Cincinnati meeting, August 1959.

in a separatory funnel along with approximately 25 ml. of distilled water and the mixture extracted with two 25-ml. portions of methylene chloride in order to remove any free alcohol. The aqueous phase was then drained into a 200-ml. volumetric flask and sufficient distilled water added through the separatory funnel to bring the solution in the flask to the mark.

Exactly 1-ml. aliquots of these extracted dilutions were then mixed with precisely 5 ml. of Porter-Silber reagent. The mixed solutions were placed in a constant temperature bath at 60° for two hours, after which the color intensity was determined against a distilled water-reagent blank at 410 mμ. Initially, all concentrations were calculated by comparison with a standard solution of hydrocortisone phosphate. Subsequently, the use of a standard was found unnecessary and all "concentrations" were obtained as a per cent of the "zero hour" sample.

RESULTS AND DISCUSSION

Order of the Reaction.—From the plots shown in Fig. 1 it is evident that the hydrolysis of hydrocortisone phosphate is first order with respect to the steroid ester under a variety of experimental conditions. This is, of course, an expected result and is quite in keeping with the order found for the hydrolyses of other organic phosphate esters (1, 3–5). The observed first-order rate constants ($k_{obs.}$) are given in Table I.

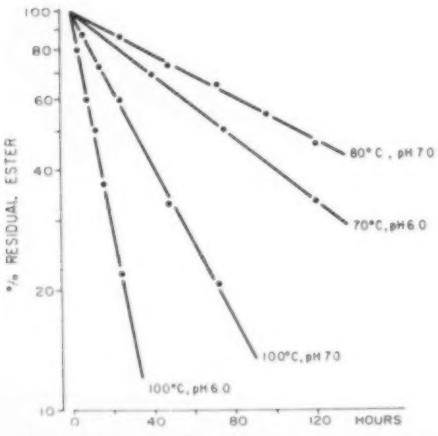

Fig. 1.—Plots showing the apparent hydrolysis of hydrocortisone phosphate to be first order with respect to the steroid ester under a variety of experimental conditions.

Unfortunately, the first-order disappearance rate found is not conclusive evidence that hydrolysis constituted the only degradative pathway. Definitive evidence towards this end could be obtained only by demonstrating the appearance of one mole of hydrocortisone alcohol for each molecule of the ester lost. Inasmuch as, under the conditions of this study, hydrocortisone alcohol degrades further into products which could not be detected by either Porter-Silber color development or by ultraviolet absorption, this molecule-for-molecule relationship could not be demonstrated.

TABLE I.—MAGNITUDES OF THE OBSERVED PSEUDO FIRST-ORDER RATE CONSTANTS

T, °C.	pH	$k_{obs.}$ – hr.$^{-1}$ × 10^3
100	7.5	13.58
100	7.0	22.72
100	6.0	63.0
91	7.5	8.15
91	7.0	14.25
91	6.0	37.86
80	7.5	3.69
80	7.0	6.36
80	6.0	17.39
70	7.5	1.92
70	7.0	3.28
70	6.0	9.24

Despite this deficiency, the reproducibility of the observed rates and their strict adherence to first order kinetics are very strong evidence of hydrolytic rather than oxidative breakdown. This evidence is further strengthened by the preliminary evidence previously cited and by the extreme care taken to exclude oxygen from the reaction systems.

pH Dependency.—As noted earlier, it was assumed that the rate of hydrolysis would increase with increasing hydrogen ion concentration. As shown in Fig. 2, this is precisely the behavior exhibited by these systems. The plots in Fig. 2 run contrary to expectations, however, in that the reaction is linearly dependent on pH in a region within which a nonlinear response would, as discussed previously, be expected.

Under the conditions of the study, significant proportions of both anionic forms of the substrate could be expected to be present at each pH level. The linear relationship seems, therefore, merely fortuitous although it is interesting to note that a similar condition prevails in the hydrolysis of monobenzyl phosphate (1).

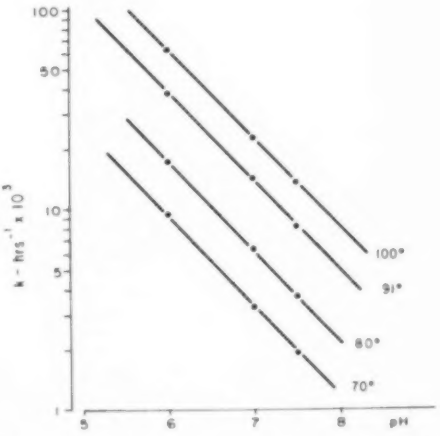

Fig. 2.—Plots indicating the effect of pH on the rate of hydrolysis of hydrocortisone phosphate. The order of reaction with respect to hydrogen ion, as determined from the slopes of these lines is 0.44.

This fortuitous linearity, while not unqualifiedly explicable at the present time, cannot be regarded as representing some simple, uncomplicated, reaction. If, for example, under the conditions of this study only one of the anionic forms of the substrate was present, the reaction would probably be second order, that is, first order with respect to each of the reactants. Since the reaction has already been demonstrated to be first order with respect to hydrocortisone phosphate and the observed rate constant is related to the apparent specific rate constant (k_{H^+}) by

$$k_{obs} = k_{H^+}(H^+)^n \qquad \text{(Eq. 2)}$$

where n is the order of the reaction with respect to hydrogen ion, the slopes of the plots in Fig. 2 should be -1.0 if the reaction was simply second order. The actual slope of these lines is, however, -0.44 in each case. This is certainly not an apparent order representative of any simple reaction.

Instead, the apparent 0.44 order with respect to hydrogen ion probably represents a weighted average order of the individual reactions characterized by the observed rate constants and given by

$$k_{obs} = k'_{H^+}(H^+)^{n'} + k''_{H^+}(H^+)^{n''} \qquad \text{(Eq. 3)}$$

where specific rate constants are the same as given in Eq. 1 and where n' and n'' are the orders with respect to hydrogen ion of the individual reactions.

If it is true that the apparent 0.44 order represents a weighted average, the linear nature of the plots in Fig. 2 permits calculation of the apparent overall specific rate constant. Upon putting Eq. 2 in its logarithmic form we can write

$$\log k_{obs} = -n\ \text{pH} + \log k_{H^+} \qquad \text{(Eq. 4)}$$

and then

$$\log k_{obs} = -0.44\ \text{pH} + \log k_{H^+} \qquad \text{(Eq. 5)}$$

thereby enabling us to calculate k_{H^+} at each temperature. These calculated average values for k_{H^+} are given in Table II.

Temperature Dependency.—The effect of temperature on the rate of hydrolysis of hydrocortisone phosphate is shown in the Arrhenius-type plots of Fig. 3. The activation energy as calculated from the slopes of these lines is 17.0 ± 1.0 Kcal./mole. The same activation energy is obtained when the calculated average specific rate constants are plotted as a function of reciprocal absolute temperature.

Since it was desirable to write a kinetic equation expressing the effect of both pH and temperature on the rate of degradation, evaluation of the "frequency factor," A, of the Arrhenius equation was necessary. This was calculated from the expression:

$$k_{H^+} = Ae^{-\Delta Ha/RT} \qquad \text{(Eq. 6)}$$

TABLE II.—CALCULATED AVERAGE VALUES OF k_{H^+} AT VARIOUS TEMPERATURES

T, ° C.	k_{H^+}
100	27.5
91	16.5
80	7.52
70	3.94

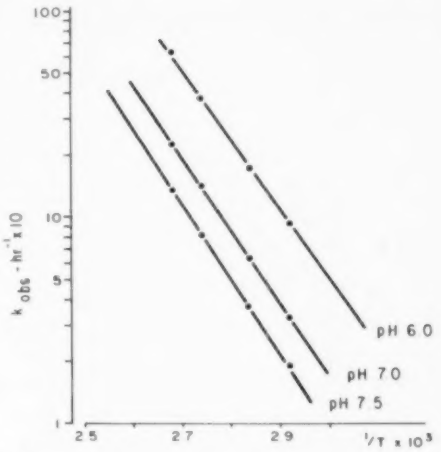

Fig. 3.—Arrhenius type plots showing the temperature dependency of the hydrolysis of hydrocortisone phosphate. The apparent activation energy as determined from the slopes of these lines is 17.0 ± 1 Kcal./mole

and was determined to have an average value 2.57×10^{11}.

The value of A was calculated in terms of k_{H^+} rather than k_{obs} because the former depends only on temperature while the latter is both temperature and pH dependent.

KINETIC EQUATION

Since, from the Arrhenius equation we can write

$$\log k_{H^+} = \log A - \Delta Ha/2.30\ RT \qquad \text{(Eq. 7)}$$

and the values for A, ΔHa, R, and T are either known or have been evaluated, our equation becomes

$$\log k_{H^+} = 11.41 - 17{,}000/4.58\ T \qquad \text{(Eq. 8)}$$

When Eq. 6 is rearranged and substituted into Eq. 8, we get the kinetic equation

$$\log k_{obs} = 11.41 - 3710/T - 0.44\ \text{pH} \qquad \text{(Eq. 9)}$$

thus expressing the effect of temperature and pH on the pseudo first-order degradation of hydrocortisone phosphate.

In practice this equation has been applied with success to formulation procedures, stability evaluation, and in permitting separation of degradations resulting from both oxidation and hydrolysis.

Although the present communication considers only the pH range 6 to 7.5, the equation presented has proved reliable in describing degradation in the pH range 5.0 to 8.5.

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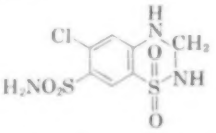
The Photometric Determination of Hydrochlorothiazide and Its Hydrolysis Product*

By C. R. REHM and J. B. SMITH

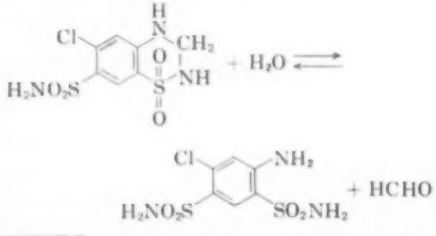
Photometric methods suitable for the routine determination of hydrochlorothiazide and its hydrolysis product, 4-amino-6-chloro-*m*-benzenedisulfonamide, have been developed. Hydrochlorothiazide undergoes an apparently reversible hydrolytic reaction in aqueous media to give 4-amino-6-chloro-*m*-benzenedisulfonamide and formaldehyde. Ultraviolet absorption measurements at 271 mμ are suitable for the determination of hydrochlorothiazide in the absence of the disulfonamide and for the determination of total hydrochlorothiazide (nonhydrolyzed plus hydrolyzed) where the extent of hydrolysis is less than 10 per cent. The disulfonamide resulting from the hydrolysis of hydrochlorothiazide is determined by reaction with nitrous acid and coupling of the diazonium compound formed with chromotropic acid. The resulting red color which is read at 500 mμ is linear in absorbance with concentration of disulfonamide. Hydrochlorothiazide and formaldehyde, as such, do not interfere, permitting the extent of degradation of hydrochlorothiazide to be accurately determined. In a similar manner, total hydrochlorothiazide is determined by alkaline saponification prior to diazotization and reaction with chromotropic acid. The intact hydrochlorothiazide is calculated from the difference between the results of the two colorimetric procedures.

HYDROCHLOROTHIAZIDE[1] is an extremely active diuretic (1–5) and has the following molecular structure:



It is a colorless crystalline compound with a melting point of 265–270° (decompn.) and a molecular weight of 297.75. Hydrochlorothiazide is very slightly soluble in water, soluble in dilute sodium hydroxide solution and methanol, and very soluble in diethyleneglycol monomethylether.

In the course of developing analytical methods suitable for the determination of hydrochlorothiazide in pharmaceutical formulations, the degradation of this compound was also investigated. Studies in these laboratories have shown that in aqueous solution hydrochlorothiazide undergoes hydrolysis to give 4-amino-6-chloro-*m*-benzenedisulfonamide and formaldehyde according to the following reversible reaction:



The instability of the $>N\text{-}CH_2\text{-}N<$ group in a number of compounds yielding formaldehyde as a hydrolysis product has been reported as early as 1907 by Votocek and Vessely (6). The hydrolysis of chlorothiazide in alkaline solution, which also yields 4-amino-6-chloro-*m*-benzenedisulfonamide and presumably formic acid (salt), has been reported by Baer, *et al.* (7). A study of the kinetics of the hydrolysis of hydrochlorothiazide, which will be presented in a subsequent paper, indicates that the equilibrium of the above reaction is far to the left.

In order to determine the stability of hydrochlorothiazide in solution and in various pharmaceutical formulations, specific analytical methods were required which would permit the extent of hydrolysis to be accurately determined. These methods are described in detail in the following sections.

EXPERIMENTAL

Apparatus and Reagents.—Ammonium hydroxide 0.5 *N*; hydroxylamine HCl 0.7% solution; hydrochloric acid 1 *N*; sodium nitrite 1% solution; ammonium sulfamate 2% solution; chromotropic acid (disodium salt) 0.5% solution, freshly prepared; sodium acetate 1 *N*; hydrochlorothiazide standard solution, 0.1 mg./ml.; 4-amino-6-chloro-*m*-benzenedisulfonamide standard solution, 0.1 mg./ml.; 100° water bath; Beckman model B spectrophotometer or other suitable spectrophotometer.

Determination of 4-amino-6-chloro-*m*-benzenedisulfonamide.—If sample is a solid, dissolve accurately weighed sample in 5 ml. of methanol or methyl cellosolve. Dilute sample with water to a volume such that final dilution contains 0.01 to 0.1 mg./ml. of disulfonamide. Diluted sample should be assayed for disulfonamide within four hours if hydrochlorothiazide is present in the sample.

* Received August 21, 1959, from the Research Department, Ciba Pharmaceutical Products Inc., Summit, N. J.
Presented to the Scientific Section, A. PH. A., Cincinnati meeting, August 1959.

[1] Esidrix is the trademark of Ciba Pharmaceutical Products Inc., Summit, N. J.

Into separate 50-ml. volumetric flasks, pipet 2 to 5 ml. of sample solution and 2 ml. of 4-amino-6-chloro-*m*-benzenedisulfonamide standard solution. To each flask add 1 ml. of sodium nitrite solution followed by 5 ml. of hydrochloric acid and mix well. Allow to stand for three to five minutes. Then add 1 ml. of ammonium sulfamate solution, mix well, and allow to stand for three minutes. To each flask add 1 ml. of chromotropic acid solution and 10 ml. of sodium acetate solution, dilute to volume with water, and mix well. Prepare a blank solution in a similar manner. Determine the absorbance of the sample and standard solutions against the blank solution in 1-cm. cells at 500 mμ in a suitable spectrophotometer.

Calculation.—Mg. disulfonamide = A sample/A standard × 0.2 × dilution factor; mg. hydrolyzed hydrochlorothiazide = mg. disulfonamide × 1.042.

Determination of "Total" Hydrochlorothiazide.—Dissolve or dilute sample with 0.5 *N* ammonium hydroxide solution so that a concentration of approximately 0.1 mg./ml. is obtained. Accurately pipet 2 ml. of sample solution and 2 ml. of standard hydrochlorothiazide solution into separate 50-ml. volumetric flasks, add 0.5 ml. of hydroxylamine HCl solution to each flask, and stopper tightly. Place the flasks in a water bath at 100° for sixty minutes. Cool the flasks to 25°, and proceed as directed under *Determination of 4-amino-6-chloro-m-benzenedisulfonamide* beginning with "to each flask add 1 ml. of sodium nitrite solution . . ."

Calculation.—Mg. "total" hydrochlorothiazide = A sample/A standard × 0.2 × dilution factor, where A sample = absorbance of sample solution and A standard = absorbance of standard solution; mg. hydrochlorothiazide (nonhydrolyzed) = mg. total hydrochlorothiazide[2] − mg. disulfonamide[2] × 1.042.

RESULTS AND DISCUSSION

Ultraviolet Absorption Spectra.—The ultraviolet absorption spectra of hydrochlorothiazide and of 4-amino-6-chloro-*m*-benzenedisulfonamide in methanol are shown in Fig. 1. Similar curves are obtained for aqueous solutions of the compounds. Absorbance measurements of methanolic and aqueous solutions of hydrochlorothiazide at the maximum of 271 mμ indicate that Beer's law is obeyed over the concentration range 1 to 10 mg./L. The $E(1\%,1$ cm.) 271 mμ of hydrochlorothiazide has been determined to be 675 ±7 (methanol) and 650 ±7 (water) in these laboratories.

It is evident from the overlapping spectral curves that ultraviolet absorption measurements are not suitable for the accurate determination of the hydrochlorothiazide moiety in the presence of the disulfonamide. Total hydrochlorothiazide (nonhydrolyzed + hydrolyzed) can be determined with fair accuracy by ultraviolet absorption measurements at 271 mμ, if the extent of hydrolysis (or contamination by the disulfonamide) is not too great. For example, if the disulfonamide were present to the extent of 10% of the amount of hydrochlorothiazide in a sample, a maximum error

[2] Values computed on basis of same total initial sample weight or volume.

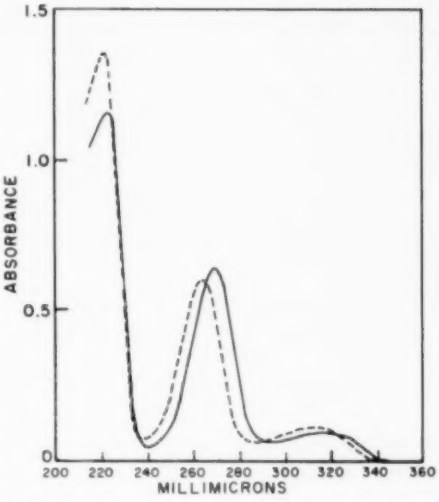


Fig. 1.—Ultraviolet spectra of hydrochlorothiazide and 4-amino-6-chloro-*m*-benzenedisulfonamide in methanol. —— Hydrochlorothiazide, - - - - - - 4-amino-6-chloro-*m*-benzenedisulfonamide.

of approximately −1% would be introduced in the determination of the sum of the two compounds by absorbance measurements at 271 mμ.

Color Reaction.—The diazotization of 4-amino-6-chloro-*m*-benzenedisulfonamide and subsequent coupling with N-(1-naphthyl)-ethylenediamine by the well-known Bratton and Marshall procedure has been used by Baer, *et al.* (7), in the determination of chlorothiazide with reportedly excellent results. Chromotropic acid (4,5-dihydroxy-2,7-naphthalenedisulfonic acid) which has been reported to give highly colored products upon reaction with diazotized *o*-aminobenzenesulfonamides (8) was used as the coupling agent in these procedures.

The diazotization reaction is complete in two minutes or less as shown by potentiometric titrations with sodium nitrite. The titrations also revealed that 2 moles of nitrous acid are consumed by one mole of the compound, indicating the reaction is apparently more complex than a simple diazotization reaction. The reaction between the diazonium compound and chromotropic acid after destruction of the excess nitrous acid results in a pink color with an absorption maximum at 500 mμ. The color development proceeds somewhat slowly in acid medium, requiring thirty to forty-five minutes for full color development. By buffering the solution to a higher pH (approximately 4.7) through the addition of sodium acetate, the color development is very rapid and absorbance values may be determined within five minutes. Absorbance values have been found to be constant over a period of several hours, although the solutions gradually attain an orange tint, apparently due to slow oxidation of the excess chromotropic acid.

The buffering of the final solution accounts, in part, for the reproducible absorbance values obtained with the standards. The relationship between absorbance and concentration is linear

over a wide range of concentration as shown in Fig. 2. The $E_{1cm}^{1\%}$ at 500 mμ in terms of original disulfonamide concentration is approximately 1,080.

In order to determine the suitability of the method for kinetic and stability studies, known samples of the disulfonamide in the presence of hydrochlorothiazide and formaldehyde were analyzed. These data which are shown in Table I indicate that the compound can be accurately determined in the presence of large molar excess amounts of hydrochlorothiazide and formaldehyde. Hydrochlorothiazide is sufficiently stable in dilute acid solution at room temperature so that no significant hydrolysis occurs during the diazotization procedure. Formaldehyde which forms an intense purple color with chromotropic acid in 80% sulfuric acid (9) apparently does not react at room temperature in acid solutions which are less than 2 *N*.

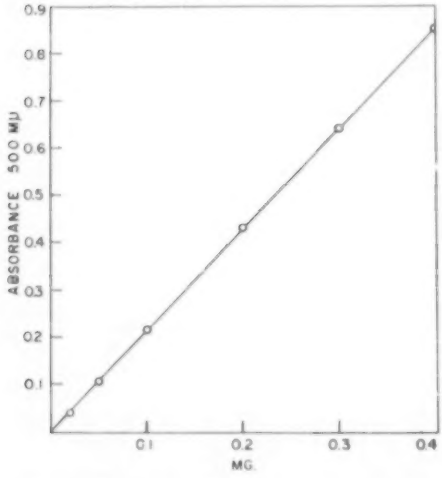


Fig. 2.—Plot of absorbance *vs.* concentration of 4-amino-6-chloro-*m*-benzenedisulfonamide after diazotization and coupling with chromotropic acid. Final volume = 50 ml.

TABLE I.—DETERMINATION OF 4-AMINO-6-CHLORO-*m*-BENZENEDISULFONAMIDE IN PRESENCE OF HYDROCHLOROTHIAZIDE AND FORMALDEHYDE[a]

Sample Composition, mg.			
Disulfonamide	Hydrochlorothiazide	Formaldehyde	Disulfonamide Found, mg.
1.00	0	...	1.01
1.00	0.50	...	1.00
1.00	1.00	...	0.99
1.00	10.00	...	1.00
1.00	50	...	1.01
1.00	...	0.01	1.00
1.00	...	0.10	1.00
1.00	...	1.00	0.99
1.00	1.00	0.10	0.99

[a] Results are averages using duplicate samples and standards.

Hydrolysis Reaction.—Studies of the decomposition of hydrochlorothiazide in aqueous solution have indicated that the hydrolysis proceeds to an apparent state of equilibrium, the extent of hydrolysis being dependent upon the concentration of drug in solution. Typical results obtained for the hydrolysis in sodium hydroxide and in ammonium hydroxide solution, as shown in Fig. 3, indicate that hydrolysis is incomplete even after ninety minutes. Refluxing in 2 *N* sodium hydroxide for sixty minutes effected complete hydrolysis, presumably by destroying the formaldehyde released, thus forcing the reaction to completion. This procedure which would be inconvenient for routine determinations did suggest, however, that the reaction could be forced to completion under milder conditions by the addition of a suitable agent to react with the formaldehyde as it was formed.

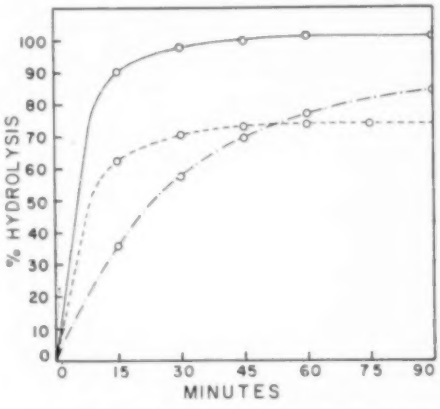


Fig. 3.—Hydrolysis of hydrochlorothiazide in alkaline media. —— 0.5 *N* NH_4OH + hydroxylamine HCl, - - - - - - - 0.5*N* NH_4OH, —·— 1*N* NaOH.

The addition of a small amount of hydroxylamine HCl to solutions of hydrochlorothiazide in 0.5 *N* ammonium hydroxide resulted in complete hydrolysis in approximately forty-five minutes at 100°, as shown in Fig. 3. A reaction time of sixty minutes was found to be suitable. Although hydroxylamine undergoes hydrolysis in alkaline solution, it is apparently sufficiently stable in ammonium hydroxide at 100° as to be available for reaction with the formaldehyde resulting from the hydrolysis of hydrochlorothiazide. Sufficient nitrite must be added in the subsequent diazotization reaction to provide an excess over that consumed by the hydroxylamine remaining.

Although analyses of hydrochlorothiazide using the disulfonamide as a standard have shown that the hydrolysis is essentially 100% over a concentration range of 0.01 to 0.1 mg./ml., it is preferable to use hydrochlorothiazide standards, carrying them through the same procedure as unknown samples.

Known samples of hydrochlorothiazide plus 4-amino-6-chloro-*m*-benzenedisulfonamide, representing hydrochlorothiazide hydrolyzed to different extents, as shown in Table II, were analyzed for

TABLE II.—DETERMINATION OF HYDROCHLOROTHIAZIDE AND 4-AMINO-6-CHLORO-*m*-BENZENEDISULFONAMIDE IN MIXTURES

Sample Composition, mg.[a]		Found, mg.[a]		% Hydrolysis	
A	B	B	C	Theory	Experimental
100.0	0	0.05	101	0	0.05
95.0	4.80	4.84	100	5	5.05
90.0	9.60	9.69	100	10	10.2
75.0	24.0	24.3	99	25	25.5
50.0	48.0	48.0	100	50	50.0
25.0	72.0	71.5	100	75	74.5
10.0	86.4	87.3	100	90	91

[a] A = hydrochlorothiazide; B = 4-amino-6-chloro-*m*-benzenedisulfonamide; A + B corresponds to 100 mg. of A hydrolyzed to extent shown; C = total of A + B determined as A; % hydrolysis (experimental) = (B × 1.042/C) × 100.

total hydrochlorothiazide and for the disulfonamide content by the procedures outlined. The per cent hydrolysis was calculated from the difference between the assay for total hydrochlorothiazide and the assay for the disulfonamide for each sample. It is necessary, of course, to convert the determined disulfonamide content of the sample to an equivalent amount of hydrochlorothiazide by multiplying by the factor 1.042, the ratio of the molecular weights. From the data shown it is apparent that hydrochlorothiazide can be determined with a precision of ±1% and that the extent of hydrolysis can be accurately determined. These procedures are applicable to the determination of hydrochlorothiazide and its stability in tablets and liquid formulations and have been used in these laboratories on a routine basis.

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The Metabolism and Central Nervous System Distribution of C-14 Carbonyl Salicylamide in the Rat*

By WILLIAM F. BOUSQUET and JOHN E. CHRISTIAN

This investigation was undertaken in order to define the central nervous system distribution pattern of C-14 carbonyl salicylamide in the rat, as a possible basis of explaining the higher level of analgetic activity of this drug as compared to other compounds of the salicylate group. The metabolic products of salicylamide in this species were identified. Various tissues were then examined for the presence of metabolic products.

NUMEROUS STUDIES (1–3) have shown salicylamide to possess favorable analgetic properties. Blood levels of this compound are low in man and animals (4), presumably due to rapid distribution to the tissues (5). In experimental animals brain concentrations of salicylamide are higher than other salicylate drugs (6).

The uptake of C-14 carbonyl salicylamide by the central nervous system of the rat has been studied at various time intervals following intraperitoneal administration of 100 mg./Kg. of salicylamide containing 3.0 μc. of C-14 activity. Samples were taken from the blood, cerebrospinal fluid, cerebral cortex, cerebellum, medulla, pituitary, hypothalamic area, and the adrenal glands at intervals of five, fifteen, thirty, sixty, and one hundred and twenty minutes after drug administration.

Various metabolic products of salicylamide have been reported in studies involving both animals and man. The predominant product observed has been salicylamide itself, either in the free state or conjugated with either sulfate or glucuronate. Studies of salicylamide metabolism in humans have failed to reveal the presence of the 5-hydroxy analog of salicylamide (gentisamide) as a metabolite (7), while this compound has definitely been established as a metabolic product in animals (8).

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Other authors (9) have reported the presence of gentisic acid in the pituitary gland of febrile rats following salicylic acid administration. Accordingly, it was of interest in this work to determine whether the pituitary plays a role in the hydroxylation mechanism leading to the formation of gentisamide from salicylamide.

The metabolic products present in the urine of both normal and hypophysectomized animals following administration of 100 mg./Kg. of salicylamide containing 3.0 μc. of C-14 activity were isolated and identified. The blood, whole brain, pituitary, cerebrospinal fluid, and the adrenal glands were examined in another group of animals in order to determine if these areas contained any metabolic products.

EXPERIMENTAL

Synthesis of C-14 Carbonyl Salicylamide.—This compound was prepared by a modification of the method of Bousquet and Christian (10), using C-14 carboxyl salicylic acid synthesized in these laboratories by Bousquet and Christian (11). The compound was determined to be both chemically and radiochemically pure before use in the animal experiments. This material was found to have a specific activity of 4.05×10^6 dpm/mg.

Central Nervous System Distribution Studies.—Male, albino rats of the Holtzmann strain were used throughout this work. These animals were maintained on Purina Laboratory Chow. At the time of use the animals weighed between 200 and 310 Gm. They were not fasted prior to injection.

Procedure.—Five animals were studied at each time interval following intraperitoneal administration of 100 mg./Kg. of salicylamide containing 3.0 μc. of C-14 activity. The drug was dissolved in 40% aqueous propylene glycol with gentle heating. The volume of solution administered to each animal was 1.0 ml. Animals were sacrificed at five, fifteen, thirty, sixty, and one hundred and twenty minutes after drug administration.

All radioactivity determinations were made with the Tri-Carb liquid scintillation spectrometer.[1] Special, low potassium content glass vials[2] were used as sample containers. Tissue samples were dissolved in 2 ml. of a 1 *M* solution of Hyamine[3] hydroxide in methanol. Correction for differences in sample counting efficiency was made using an internal standard technique (12).

After injection the animals were placed in individual cages. One minute before expiration of the time period allowed for uptake of the drug, the animal was removed from the cage, anesthetized lightly with ether, and a sample of cerebrospinal fluid withdrawn by puncture of the cisterna magna with a 27-gauge needle fitted with a length of polyethylene tubing. The fluid was allowed to flow to a predetermined height in the tubing such that about 40 mg. was obtained. In some cases it was necessary to apply suction to the tubing. The fluid sample was transferred to a sample vial containing 2.0 ml. of Hyamine solution, which was capped and set aside.

The animal was then decapitated and the free flowing blood collected in a three-inch evaporating dish containing 10 drops of a saturated solution of ammonium oxalate. This was transferred to a 15-ml. tube and centrifuged for twenty minutes. Three 25-λ plasma samples were transferred to sample vials containing Hyamine as described for the CSF samples.

The brain was perfused through the carotids using 15 ml. of 0.85% saline. The skull was opened, the brain removed intact, rinsed rapidly with saline, and set aside on a piece of paper toweling moistened with saline. The pituitary was then removed and treated likewise. The adrenal glands were removed and dissected free of fat.

The pituitary and the adrenals were weighed and transferred to sample vials containing 2.0 ml. of Hyamine. Two samples of the medulla, cerebellum, cerebral cortex (no white matter), and the hypothalamic area from each animal were dissected out, weighed, and transferred to sample vials. All tissue samples taken were less than 40 mg. in weight.

When all tissue samples from a given group of animals had been taken, they were set aside in the sample vials to allow dissolution to begin. After twenty-four hours all samples with the exception of the plasma and CSF were placed in an oven and heated at 60° until the tissue dissolved. This usually required not over seventy-five minutes if the caps were removed from the vials prior to heating. It is imperative that the temperature not exceed 60°. Higher temperatures cause the solutions to become dark brown in color and counting efficiency is greatly impaired. Upon completion of tissue dissolution the vials were removed from the oven, cooled to room temperature, 15 ml. of liquid phosphor solution[4] added, and the samples counted in the Tri-Carb.

Results.—The results obtained in the central nervous system distribution study are shown graphically in Fig. 1. Activities are expressed on a wet weight basis. Maximal central nervous system uptake of C-14 carbonyl salicylamide in the rat is seen to occur between five and fifteen minutes following administration of the labeled compound. No well-defined peak is seen for the blood, but higher concentrations are evident between thirty and sixty minutes. Between sixty and one hundred and twenty minutes there appears to be little change in concentration in any area but the hypothalamus.

The concentration ratio between the cerebrospinal fluid and the plasma reached unity in twelve minutes, indicating rapid transport from the plasma. It is of interest that salicylic acid, a substance of similar molecular structure, requires over six hours to reach equivalent concentration in the cerebrospinal fluid of rabbits (13).

[1] Packard Instrument Co., LaGrange, Ill.

[2] Five-dram, crystallite vial, #A-3305, T. C. Wheaton Co., Millville, N. J.

[3] A high molecular weight quaternary ammonium compound which dissolves animal tissue. Supplied by the Rohm and Haas Co. It was converted from the hydrochloride to the free base by treatment with sodium hydroxide.

[4] Prepared by dissolving 102 Gm. of naphthalene, 6.5 Gm. of PPO (2,5-diphenyloxazole), and 0.13 Gm. of POPOP (1, 4-di-[2-(5-phenyloxazoyl)]-benzene) in a mixture of xylene, 500 ml.; dioxane, 500 ml.; and absolute ethanol, 300 ml.

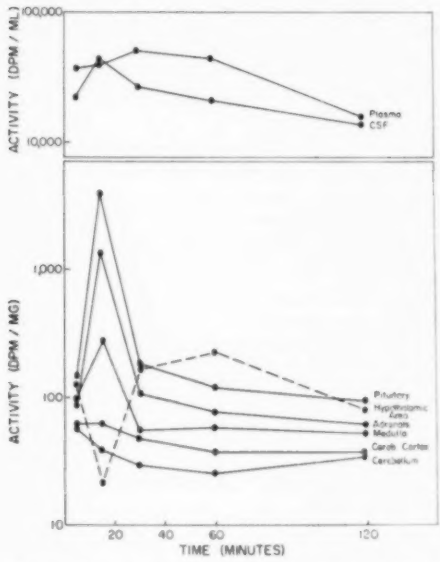

Fig. 1.—Central nervous system distribution of C-14 carbonyl salicylamide in the rat.

The low value obtained for the fifteen-minute sample from the hypothalamic area was checked using data from three additional animals. The results obtained (21 dpm *vs.* 23 dpm) indicate that this low value was not due to error in sample preparation.

Metabolism of C-14 Carbonyl Salicylamide.—*Procedure.*—The animals used in this study were not fasted prior to use and were watered but not fed during the time of urine collection. Administration of 100 mg./Kg. of salicylamide containing 3.0 μc. of C-14 activity was made by intraperitoneal injection. Seven animals were studied.

Immediately after injection, each animal was placed in a glass metabolism chamber similar to that described by Roth, *et al.* (14), the urine being collected separately from the feces over a twenty-four hour period. The collection flask contained 1.0 ml. of glacial acetic acid to prevent alkaline oxidation of any diphenolic metabolites formed.

At the end of the collection period, the urine was filtered and an aliquot taken for radioactivity analysis. The remainder of the urine was acidified with concentrated sulfuric acid and immediately extracted with three volumes of ether in three divided portions. The ether extract was reduced in volume by evaporation so that 10 λ of extract was equivalent to approximately 100 λ of whole urine. An aliquot of this extract was taken for analysis by two-dimensional paper partition chromatography. Solvent pairs employed were isopropanol:water, 100:10 (solvent A); and benzene:glacial acetic acid, 8:2 (solvent B). Whatman No. 1 paper was used. After development the papers were examined under ultraviolet light to locate gentisates, and then sprayed with ferric nitrate solution to locate salicylates. Another aliquot of this extract was taken for radioactivity analysis.

The degree of separation achievable between the suspected metabolic products of salicylamide using these solvent pairs is shown in Table I. In deter-

TABLE I.—R_f VALUES OF POSSIBLE SALICYLAMIDE METABOLITES

Compound[a]	$R_f \times 100$ Solvent A[b]	Solvent B[c]
Salicylic acid	51	95
Salicylamide	88	95
Gentisic acid	23	23
Gentisic acid amide	86	14

[a] Ten micrograms of each compound used. Running times for a 20-cm. rise were approximately eight hours for solvent A and three hours for solvent B at room temperature.
[b] Isopropanol:water, 100:10.
[c] Benzene:glacial acetic acid, 80:20.

mining the fluorescence of gentisates it is important to dry the papers thoroughly until the odor of acetic acid is no longer perceptible.

The acidified urine remaining was transferred to a 50-ml. round-bottom flask fitted with a reflux condenser and heated in a boiling water bath for twenty-five minutes to hydrolyze any conjugates present. Upon cooling to room temperature, this was then extracted with ether and concentrated as described above. Samples of this extract were both chromatographed and analyzed for radioactivity.

A control was run wherein the suspected metabolic products of salicylamide containing the amide function (salicylamide and gentisic acid amide) were added to urine, the urine then hydrolyzed, extracted, and chromatographed as above. No breakdown to salicylic or gentisic acids was detected under the hydrolysis conditions employed. Another control urine was run and carried through the hydrolysis and extraction procedures. Upon chromatography of this urinary extract, no materials were present which fluoresced under ultraviolet light[5] or gave a color reaction with ferric nitrate solution.

The experiments were repeated using hypophysectomized animals[6] in order to determine if the pituitary plays a role in the hydroxylation mechanism. These animals were maintained on a diet consisting of: Canned dog food, 480 Gm.; skim milk, 480 ml.; and 2 slices of crumbled whole-wheat bread, the ingredients being well mixed. Five per cent dextrose was supplied as drinking water (15). Administration of the drug, collection, and treatment of urine samples were identical to that described for the normal animals, except that the urine was immediately hydrolyzed as it was of interest only to determine if any gentisic acid amide was present.

Results.—Radioactivity determinations on the whole urine of both the normal and hypophysectomized animals indicated that about 96% (range 91–99%) of the administered radioactivity was present in the urine after twenty-four hours. Radioactivity present in the ether extract of the unhydrolyzed urine from normal animals showed

[5] Blak-Ray long wave ultraviolet light, model B-50, Ultra-Violet Products Inc., South Pasadena, Calif.
[6] Sprague-Dawley strain. Male, 250 Gm. Supplied by the Hormone Assay Laboratories, Chicago, Ill.

that about 3% (range 1.7–7.4%) of the compound was excreted in the free form, assuming that extraction was complete and that only free salicylamide was extracted.

Chromatographic analysis of the ether extract of the unhydrolyzed urine from normal animals revealed the presence of only one spot corresponding to free salicylamide. In two animals, an additional spot was present upon spraying with ferric nitrate. This was probably salicyluric acid, although a definite identification was not made. No gentisic acid amide was found in these extracts indicating that any of this compound formed *in vivo* is not excreted in the free form.

Chromatography of the ether extracts of the acid hydrolyzed urines showed the presence of two spots, one corresponding to salicylamide and one to gentisic acid amide. These were observed in both normal and hypophysectomized animals, thus suggesting that the pituitary does not play a role in the hydroxylation mechanism. In order to confirm the presence of gentisic acid amide in the extracts this compound was added to the extract and chromatographed again. A single fluorescent spot was present which was found to contain the radioactivity.

It was of interest to determine the relative amounts of salicylamide and gentisic acid amide present in the urine after hydrolysis. Accordingly, the chromatogram spots containing these materials were cut from the paper and analyzed for their radioactivity. These data indicate that between 5 and 30% of the excreted radioactivity is associated with gentisic acid amide.

It was not possible to show the presence of either salicylic acid or gentisic acid in the urinary extracts. Thus it appears that the amide function is not broken down during salicylamide metabolism in this species. The biostability of the amide function is well recognized (16, 17).

Tissue Metabolites of C-14 Carbonyl Salicylamide. It was of interest to determine if the hydroxylated metabolite of salicylamide which presumably (18, 19) is formed in the liver of experimental animals, is returned to the general circulation and distributed to the body tissues. The plasma, cerebrospinal fluid, pituitary gland, adrenal glands, and the brain were examined for the presence of metabolites.

Procedure.—Male rats were dosed as previously described. Fifteen minutes after drug administration a sample of cerebrospinal fluid was withdrawn from each anesthetized animal. A 10-λ sample of CSF was chromatographed using the solvent systems described for the urine samples.

At twenty minutes the animals were sacrificed by decapitation, the blood being collected with oxalate. The pooled blood from three animals was centrifuged and 4.0 ml. of clear plasma removed with a pipet. To this was added 2.0 ml. of a 10% solution of trichloroacetic acid and the sample again centrifuged. Four milliliters of clear liquid was withdrawn. This was extracted with three 10-ml. portions of ether and concentrated so that 20 λ of extract was equivalent to approximately 100 λ of plasma. Twenty-λ portions of the extract were chromatographed.

The brains were pooled and homogenized with 7 ml. of saline. The total volume of homogenate was about 13 ml. One milliliter of concentrated hydrochloric acid was added and the homogenate extracted with five 25-ml. portions of ether. The extract was concentrated to 1 ml., and 15-λ portions chromatographed.

The pituitaries from the three animals were transferred to a test tube containing 1.0 ml. of a 500 mg. % solution of pepsin in 0.1 *N* hydrochloric acid. This was heated at 38° for eighteen hours. The fine suspension produced was extracted with three 10-ml. portions of ether, the extract concentrated to 100 λ, and 15-λ aliquots chromatographed.

The adrenals were treated in the same manner as the pituitary glands.

Results.—Examination of the chromatograms of the tissue extracts revealed the presence only of salicylamide. This was found in all of the extracts. Under ultraviolet light no fluorescence was found in the area where gentisic acid amide would be present. In order to confirm the finding that gentisic acid amide is not present in the tissues following salicylamide administration, the chromatograms of the tissue extracts were exposed to Eastman-Kodak "No-Screen" X-ray film for a period of one month. This technique would serve to indicate the presence of minute amounts of gentisic acid amide which would not be detectable by conventional procedures. At the end of the exposure period no darkening of the film was observed.

DISCUSSION

The finding of highest activity in the pituitary gland following administration of C-14 carbonyl labeled salicylamide is in agreement with reports of other authors who found the same to be true in the case of labeled aspirin (20). Further, these data support an earlier report of Bousquet and Christian (10) that whole brain concentrations of salicylamide are much higher than plasma concentrations.

The greatest positive slope of the plasma curve coincides in time with the greatest negative slope of the tissue curves. Assuming that complete absorption had taken place, this would indicate that peak plasma levels cannot be reached until the compound is removed from the tissues.

The finding of highest activity in the pituitary cannot readily be explained. Although the pituitary is thought to lie outside the blood-brain barrier, the possibility exists that this area selectively concentrates the drug. The high uptake of radioactivity by the pituitary warrants further study regarding the mechanism of action of antipyretic-analgetic drugs of this type. Other authors (9) report that gentisic acid is present in the pituitary gland of febrile rats following administration of aspirin or salicylic acid. It should be emphasized that the animals used in this study were not in the febrile state. However, the finding of identical metabolites in normal and hypophysectomized animals would seem to rule out any normal hydroxylating function of this gland. The possibility does exist that the disposition of hydroxylated metabolites would be different in febrile animals.

SUMMARY AND CONCLUSIONS

C-14 carbonyl labeled salicylamide was synthesized from C-14 carboxyl salicylic acid by a modification of the method of Bousquet and Christian. Chemical and radiochemical purities of the labeled compound were determined by paper partition chromatography and autoradiography.

The distribution of C-14 carbonyl salicylamide in the central nervous system, adrenals, and plasma of the rat was studied at time intervals of five, fifteen, thirty, sixty, and one hundred and twenty minutes following intraperitoneal injection of 100 mg./Kg. of salicylamide containing 3.0 μc. of C-14 activity. Tissues taken for radioactivity analysis include the medulla, cerebellum, cerebral cortex, hypothalamic area, pituitary gland, cerebrospinal fluid, adrenal glands, and the plasma. The pituitary gland showed the greatest uptake of radioactivity of all tissues examined. Peak activity occurred at fifteen minutes for the pituitary, medulla, cerebrospinal fluid, and the adrenals. The hypothalamic area did not show a peak until sixty minutes. No well-defined peak was seen for the cerebellum and the cerebral cortex. The plasma did not exhibit its highest activity until thirty minutes, this time appearing to coincide with removal of the drug from the tissues.

The metabolism of C-14 carbonyl salicylamide was studied in normal and hypophysectomized rats. Following hydrolysis and ether extraction of the urine, paper chromatographic separation indicated the presence of salicylamide and gentisic acid amide. Free salicylamide was shown to be present in the unhydrolyzed urine while gentisic acid amide is apparently excreted entirely as a conjugate in this species. Gentisamide was shown to occur in the urine to the extent of between 5 and 20 per cent.

The products obtained in the urine of the hypophysectomized animals were identical to those in the normal animals, indicating that the pituitary gland does not play a role in the hydroxylation of salicylamide.

In another group of animals the brain, pituitary gland, cerebrospinal fluid, adrenals, and plasma were examined for the presence of metabolic products. Paper chromatographic separation of ether extracts from these tissues showed the presence only of salicylamide. No gentisic acid amide was found in the tissues studied.

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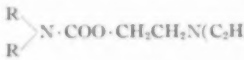
Studies on Local Anesthetics XXV[1] (Physicochemical Properties of Drugs V[2])*

By ALEŠ SEKERA† and ČENĚK VRBA‡

The authors have studied the anesthetic activity of thirty diethylaminoethyl esters of substituted carbamic acids by surface and infiltration techniques and have attempted to correlate this activity with certain physicochemical properties, namely: surface tension activity, adsorbability, liposolubility, ability to coagulate colloids, and basicity of these compounds. The results obtained have been evaluated tabularly, graphically, and statistically. At the same time a summary has been given of the earlier results and they have been included in the discussion.

STUDIES OF THE RELATIONS between physicochemical properties and local anesthetic activity are fairly numerous (1). Their results have not only contributed to the elucidation of the mechanism of action of this group of drugs but also furnished some valuable hypotheses for the choice of the structures of new synthetized substances. It is essentially for this reason that we have given attention to these relations in our studies of the new local anesthetics of the series of basic carbamates.

On 30 substances of general formula I (R = H, alkyl, phenyl, tolyl, xylyl, alkoxyphenyl, naphthyl) (2) we have studied five physicochemical properties: surface tension (3), displacement



I

adsorbability (4), liposolubility (5), ability to coagulate colloids (6), and basicity (5). Easily accessible from the experimental point of view, they were chosen because of the importance which is attributed to them according to different theories of the mechanism of action of local anesthetics. They were compared with the relative activities of three kinds of anesthesia: surface, infiltration, and conduction (7), calculated as quotient of the durations of the complete anesthesia after application of the aqueous equimolar solutions of the substances studied and of those of the standards utilized (method A). The results obtained were graphically and statistically evaluated.

In our subsequent studies we have worked out a more precise method of testing surface and infiltration anesthetic activity (8) based on the determination and the comparison of the molar concentrations of the compounds studied, which have the same activity as the selected concentrations of the standards (method B). We have abandoned the study of conduction anesthesia because this type has similarities with infiltration anesthesia and because the method used was inaccurate. With the aid of this new method we have again studied the active substances of the series mentioned. The comparison of the new values of the relative activities thus obtained with the values of the physicochemical measurements already determined constitute the subject of this communication. In view of a better comprehension of the results given we are presenting also the principles of the physicochemical methods utilized, with references to our earlier papers which contain detailed descriptions of them. This paper also summarizes some of our preceding results, which are presented in Tables I to III and are discussed and compared with our new results.

EXPERIMENTAL

Relative Surface Tension Measurements (3).—The relative surface tension activity was expressed by the relative values of the surface tension of 0.05 *M* aqueous solutions, buffered to pH 7.4. The measurements were made with Traube's stalagmometer, the laboratory temperature being 18 to 20°. The relative values were calculated according to the formula

$$\psi = \frac{a}{x} \cdot s$$

where a = number of drops of water (with the stalagmometer used, 31.89), x = number of drops of the 0.05 *M* solution of the anesthetic, and s = density 20/4 (pycnometer method) of this solution.

Relative Displacement Adsorbability (4).—One hundred milligrams (±0.2 mg.) of active charcoal (Ostakol, Spolek pro chemickou a hutní výrobu, Bohumín, Czechoslovakia) was shaken for an hour with a mixture of the aqueous solutions of 25 cc. of the anesthetic 0.04 *M* and 25 cc. of methylene blue

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[1] Paper XXIV: *Arch. intern. pharmacodynamie*, in press.
[2] Paper IV: *Ann. pharm. franc.*, **16**, 525(1958).

TABLE I.—PHYSICOCHEMICAL PROPERTIES OF INACTIVE DIETHYLAMINOETHYLESTERS OF SUBSTITUTE CARBAMIC ACIDS

No.	R_1	R_2	Relative Surface Tension Non-buffered	Relative Surface Tension Buffered pH 7.0	Relative Displacement Adsorbability	Limit Molar Coagulating Concentration	Relative Liposolubility	pKa
S 10	H	H	1.013	1.021	3.56	1.503	0.28	8.92
S 1	CH_3	H	1.009	1.017	3.08	0.887	0.28	8.95
S 2	C_2H_5	H	1.005	1.007	3.42	0.887	0.24	8.94
S 21	CH_3	CH_3	1.008	1.003	2.54	1	0.36	8.92
S 19	Δ^2-C_2H_5	H	1.007	0.998	2.66	0.4	0.24	(8.82)
S 3	C_3H_7	H	0.997	0.980	5.24	0.25	0.96	8.95
S 5	$(CH_3)_2CH$	H	0.998	0.982	4.24	0.4	0.96	8.91
S 4	C_4H_9	H	0.979	0.939	7.26	0.094	2.24	8.93
S 7	$CH_3CH_2CH(CH_3)$	H	0.982	0.956	7.26	0.122	0.24	8.95
S 8	$(CH_3)_3C$	H	1.004	0.995	3.94	0.5	0.28	8.93
S 22	C_2H_5	C_2H_5	0.978	0.934	4.62	0.375	0.6	8.89
S 23	$-CH_2CH_2CH_2CH_2CH_2-$		0.987	0.956	5.16	0.225	5.6	8.87
S 24	$-CH_2CH_2 \cdot O \cdot CH_2CH_2-$		1.006	0.998	3.26	1	0.4	8.74
S 9	C_5H_{11}	H	0.958	0.889	10.48	0.075	11.88	8.97

at 200 mg. %. After sedimentation the solution was filtered on a desorbed filter JENA 3G4; 30 cc. was taken out and in the rest of the solution the methylene blue was determined colorimetrically. The relative values of the adsorbent power are expressed directly by the final concentrations of methylene blue found in the experiments.

Relative Liposolubility (5).—Twenty-five cubic centimeters of the 0.01 *M* solution of the anesthetic, saturated with ether, was diluted with 50 cc. of water saturated with ether. Then were added 10 cc. of ether saturated with water and 25 cc. of the phosphate buffer pH 7.4 saturated with ether. The mixture was shaken for ninety minutes. Five cubic centimeters of the ethereal layer was pipeted out, evaporated, and the residue was weighed after drying in a desiccator. The final values indicate the quantities of substances extracted in 10 cc. of ether and are expressed in percentage of the base weighed.

Ability to Coagulate Colloids (6).—The ability to coagulate colloids was studied by determination of the limit molar concentrations of the anesthetics, coagulating the aqueous ethanol solution of Mastix, buffered at pH 7.0.

Basicity (5).—The basicity was studied by determination of the dissociation constants pKa. These were calculated from the pH of the aqeuous solutions of the anesthetics of known concentrations of dissociated and nondissociated base.

PHARMACOLOGY

Determination of Local Anesthetic Activity.—*Method A.*—The relative activities in surface anesthesia (rabbit's cornea, cocaine 0.05 *M* as standard), infiltration anesthesia (guinea pig, intradermal application, procaine 0.05 *M* as standard), and conduction anesthesia (frog's ischiatic nerve, procaine 0.01 *M* as standard) were calculated as quotients of the durations of the complete anesthesia after application of the equimolar solutions of the substances studied and standards utilized.

Method B.—The relative activities in surface anesthesia (rabbit's cornea, cocaine 0.01 *M* as standard) and infiltration anesthesia (guinea pig, intradermal application, procaine 0.02 *M* as standard) were calculated as quotients of the molar concentrations of the standards chosen and of the substances studied, having the same activity.

The detailed description as well as the evaluation of these two methods has been published elsewhere (7, 8). The final results presented in Tables I and II are taken in part from our earlier papers (2, 9) for purposes of comparison.

STATISTICS[1]

The statistical evaluation of the relations studied was made by the calculation of Spearman's correlation coefficients (10).

The calculations were not made in the case of basicity because even by direct comparison no relation between the pKa and the anesthetic activities was found.

DISCUSSION AND SUMMARY

The comparison of local anesthetic activity and five physicochemical properties (surface tension activity, adsorbability, liposolubility, ability to coagulate colloids, and basicity) has been made in the series of 30 basic carbamates. The results of these studies have been presented tabularly (Tables I and II), graphically (Figs. 1 and 2), and statistically (Table III). In the tabular presentation the inactive substances were classified according to molecular weight and the active substances according to the place of substitution of the benzene nucleus of carbanilic acid. In the graphic presentation the active substances are arranged according to their activities.

The following relations between chemical structure, anesthetic activity, and physicochemical properties appear to exist:

1. The parallelism between the anesthetic activity and the physicochemical properties studied is apparent even in direct comparison. The physicochemical properties of the inactive aliphatic derivatives (Table I) differ clearly from the properties of the active aromatic derivatives, the latter manifesting a surface tension activity, an adsorbability, a liposolubility, and an ability to coagulate colloids distinctly higher.

[1] The authors wish to thank Dr. B. Chalupa for calculation of the correlation coefficients.

TABLE II.—RELATIONSHIPS BETWEEN THE ANESTHETIC ACTIVITIES AND THE PHYSICOCHEMICAL PROPERTIES OF THE ACTIVE DIETHYLAMINOETHYLESTERS OF SUBSTITUTED CARBAMIC ACIDS

No.	R	R₁	Relative Activity Calculated from the Duration of the (Method A) Surface Anesthesia	Infiltration Anesthesia	Conduction Anesthesia	Inverse Concentrations (Method B) Surface Anesthesia	Infiltration Anesthesia	Relative Surface Tension Non-buffered	Buffered pH 7.0	Relative Displacement Adsorbability	Limit Molar Coagulating Concentration	Relative Liposolubility	pKa
S 11	C_6H_5	H	0.32	1.4	2.8	0.3	1.2	0.993	0.943	28.2	0.0112	10.8	8.81
S 12	α-$C_{10}H_7$	H	0.52	3.3	2.5	0.67	3.4	0.921	0.892	65.8	0.00816	21.6	8.76
S 25	C_6H_5	C_2H_5	0.8	4	1.9	1.4	3.6	0.899	0.661	45.4	0.00802	30.2	8.76
S 31	2-$CH_3C_6H_4$	H	0.46	1.1	2.4	0.54	1	0.989	0.929	24.3	0.0125	9.8	8.81
S 35	2-CH_3O-C_6H_4	H	0.5	1.6	2.6	0.33	1.7	0.988	0.931	30	0.033	11.4	8.84
S 38	2-C_2H_5O-C_6H_4	H	0.72	3	3.8	0.31	2.1	0.967	0.856	38.9	0.0175	20.8	8.82
S 44	2-C_3H_7O-C_6H_4	H	3	3	2.6	21	42	0.926	0.440	68.8	0.00983	38.9	8.68
S 36	3-CH_3O-C_6H_4	H	0.13	1.5	0.8	0.13	0.64	0.976	0.909	43.2	0.0175	11.1	8.77
S 32	3-$CH_3C_6H_4$	H	0.43	1.6	1.6	0.26	1.33	0.952	0.852	37.1	0.0125	18.21	8.84
S 39	3-C_2H_5O-C_6H_4	H	0.82	2.4	3.2	0.46	1.7	0.913	0.746	50	0.010	20.6	8.76
S 45	3-C_3H_7O-C_6H_4	H	4	2.8	1.1	30	70	0.881	0.483	83.2	0.00218	39.4	8.68
S 37	4-CH_3O-C_6H_4	H	0.8	0.8	0.8	0	0.86	0.983	0.941	35	0.055	7.8	8.81
S 40	4-C_2H_5O-C_6H_4	H	0.13	1	0	0.02	0.87	0.938	0.802	46.4	0.0195	13.1	8.80
S 34	2,4-$(CH_3)_2C_6H_3$	H	0.27	1.2	1.1	0.21	1.34	0.939	0.799	21.9	0.0175	14.2	8.82
S 33	4-$CH_3C_6H_4$	H	0.31	1.2	1.7	0.03	1.24	0.947	0.828	25.2	0.0196	16.6	8.85
S 46	4-C_4H_9O-C_6H_4	H	1.3	2.3	1.3	1.4	3	0.928	0.482	80	0.00188	37.9	8.68

[a] The substance being prepared as the base, the pKa was not determined. In the case of the isomeric β-naphthyl derivative pKa = 8.67.

In the case of the aromatic derivatives the relationships appear still more distinctly in the groups formed according to the position of substituents in the benzene nucleus of carbanilic acid (Table II); in the homologous series of the alkoxyl derivatives they approach the quantitative relations.

It seems interesting to note that with the formation of the groups according to the substituents (position isomers) the relations studied are partially decreased. This phenomenon might be associated with the supposition that the position isomery influences relatively little the processes of adsorption, the liposolubility, and the ability to coagulate colloids, and intervenes more noticeably in the other physicochemical properties, e. g., the stability of the carbamic group of carbanilic acid (metabolism), which depends upon the distribution of the electrostatic charge of the molecule varying with the position of the substituent in the benzene nucleus.

2. It is striking to note the parallelism of the determined values of surface tension activity, adsorbability, liposolubility, and the ability to coagulate colloids when compared among themselves. The relation between surface tension activity and adsorbability is easily explained, for it is a question of the same physicochemical process differing only in the nature of the phases. Also understood is the affinity of these adsorbabilities at the contact of two phases with the liposolubility, affinity already pointed out by Traube in the case of narcotics (11).

The parallelism of the limit concentrations coagulating the Mastix colloidal solution seems to indicate that in the series studied this physicochemical property is close to the processes of adsorption and less dependent on the electrostatic charge of the molecule (pKa). This fact concurs also with the relations found in the group of the narcotics (12).

3. A relationship between anesthetic activity and basicity was not found. This probably results from the fact that the pKa of the substances studied differ only very little from each other and that they have rather high values; at pH 7.4 of the biolog cal substrate all the substances are dissociated within 96-98%. The influence which they exercise on the other physicochemical properties is slight because of these very feeble differences.

4. The results obtained by the graphical representation correspond to those found by use of the tabular classification; the relationships between the anesthetic activity and the

TABLE III.—STATISTICAL EVALUATION OF THE RELATIONS STUDIED (SPEARMAN'S CORRELATION COEFFICIENT)

Physicochemical Property	Relative Activity Calculated from the Duration of the (Method A)			Relative Activity Calculated from the Equieffective Concentrations (Method B)	
	Surface Anesthesia	Infiltration Anesthesia	Conduction Anesthesia	Surface Anesthesia	Infiltration Anesthesia
Surface tension activity (nonbuffered solutions)	0.745 ± 0.057	0.776 ± 0.052	0.601 ± 0.083	0.759 ± 0.054	0.798 ± 0.047
Surface tension activity (buffered solutions)	0.852 ± 0.035	0.817 ± 0.043	0.716 ± 0.063	0.853 ± 0.035	0.869 ± 0.031
Displacement adsorbability	0.880 ± 0.029	0.879 ± 0.029	0.742 ± 0.058	0.865 ± 0.033	0.892 ± 0.026
Distribution coefficient	0.882 ± 0.029	0.878 ± 0.030	0.767 ± 0.053	0.881 ± 0.029	0.914 ± 0.021
Ability to coagulate colloids	0.905 ± 0.023	0.886 ± 0.028	0.817 ± 0.043	0.912 ± 0.022	0.913 ± 0.021

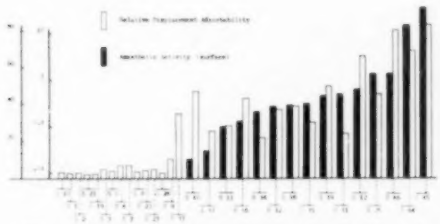

Fig. 1.—Relationship between the relative displacement adsorbability and the surface anesthetic activity (method B).

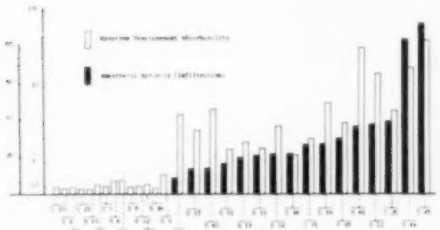

Fig. 2.—Relationship between the relative displacement adsorbability and the infiltration anesthetic activity (method B).

physicochemical properties are distinct without being quantitative. As an example we present the graph of the relations between the surface anesthetic activity (method B) and the displacement adsorbability (Figs. 1 and 2).

5. Statistical evaluation showed a clearly positive relation between the anesthetic activity and the physicochemical properties, which follows from the high values of the correlation coefficients (Table III). The distinctly lower values in the case of conduction anesthesia (method A) are probably due to the fact that the method utilized furnished results difficult to reproduce and rather dispersed.

From the statistical results it seems also that the ability to coagulate colloids is an important property in the mechanism of local anesthetic action. The increase of the correlation coefficients in the series is striking: surface tension activity (nonbuffered solutions) <surface tension activity (buffered solutions) <displacement adsorbability, which seems to concur with the progressively closer relationship between these model experiments and the process of adsorption taking place in the organism.

In tabular and even in graphical comparison the conclusions obtained with the new method of testing (method B) differ very little from those deduced from the pharmacological results obtained with the earlier method of testing (method A). Only statistical evaluation showed the distinct difference. The correlation coefficients of the physicochemical properties with the relative activities obtained by the new method are, in general, higher. This results, probably, from the greater precision of the pharmacological method (see, on the other hand, the low values of correlation coefficients in the case of the inexact method of conduction anesthesia, already mentioned).

The theoretical results obtained have also found a practical use in the choice of the structures of new synthetized substances. They made evident the advantage of alkoxylation which increases the liposolubility and the other physicochemical properties studied and similarly the local anesthetic activity. This influence grows with the prolongation of the carbon cham of alkoxyl. The k owledge of this fact, seen already in the studies of the methoxyl and ethoxyl derivatives S 35 to S 40, led us to the synthesis of the butoxyl derivatives S 44 to S 46. Only later these substances were included in the studies of physicochemical properties. In the framework of our following research the same reason led us to the profound study of the influence of alkoxylation, aralkoxylation, and aroxylation in the series of basic carbamates and anilides. In the course of these studies we have been able to prepare several relatively active substances with a fairly low order of toxicity which were, in part, the subject of our previous publications (13–15).

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The Design of a Continuous Recording *In Vivo* Method of Measuring Sensible Perspiration Over a Limited Area*

By WILLIAM J. O'MALLEY† and JOHN E. CHRISTIAN

The objective of this study was to develop a continuous recording, *in vivo* method of measuring sensible perspiration over a limited area. The study required the design and construction of a suitable electrical circuit and electrolytic cell containing a methanol–acetone–oxalic acid mixture which was sensitive to small amounts of water. The immediate desired application was for the evaluation of antiperspirant preparations. The perspiration was collected by passing dry nitrogen gas through a skin cell which enclosed a small area of skin and delivered the moisture-laden gas to the electrolytic solution. Two such systems were combined through suitable electrical connections to compare, simultaneously and continuously, two adjacent areas of skin.

THE MAIN OBJECTIVE of this study was to develop a continuous recording, *in vivo* method of measuring sensible perspiration over a limited area of the body. The immediate desired application was for the evaluation of antiperspirant preparations; however, the method should also be applicable to the evaluation of any drug which affects the rate of perspiration flow.

The electrical conductivity of mixed organic solvents has been used to measure water vapor (1–4). Such electrical conductivity measurements require the use of a d. c. Wheatstone bridge as the means of measuring the change in the resistance of the electrolytic cell as water is added (5–7). Bright platinum electrodes (8) are required in the presence of organic solvents such as methanol, acetone, and 1,4-dioxane to prevent redox reactions at the electrodes. Weak electrolytes have the advantage, over strong electrolytes, of being more soluble in the organic solvents (9). In addition, weak electrolytes are not dissociated in the organic solvents and are only slightly dissociated in water; therefore, concentration in these systems is not a critical factor (10).

EXPERIMENTAL

This study required the design and construction of suitable circuits and electrolytic cells containing a methanol-acetone-oxalic acid mixture which is sensitive to small amounts of water. The perspiration was collected by passing dry nitrogen gas through a skin cell which enclosed two adjacent areas of skin and delivered the moisture-laden gas to the electrolytic solutions.

The initial experimental phase of this study involved the design and development of the continuous recording instrumentation which permitted the measurement of small quantities of water vapor (sensible perspiration) in gases. To permit the study of two adjacent areas, two separate systems were combined; one gaseous, to carry the moisture from the skin to the electrolytic cell, and the other electrical, to measure the moisture added to the

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electrolytic cell. The electrolytic solution was sensitive to very small (0.010-ml.) quantities of water. The water thus collected was measured by noting the decrease in electrical resistance using a d. c. Wheatstone bridge. Since it was desirable to compare two adjacent areas continuously and simultaneously, it was necessary to have two electrolytic cells, two nitrogen supply lines, and two bridge circuits. The two bridges were connected to a single point recorder for continuous, simultaneous recording. Figure 1 gives a schematic diagram of the dual system used in these studies.

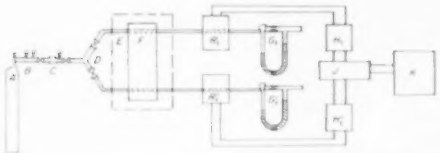

Fig. 1.—A schematic of the dual system. *A*, dry nitrogen gas; *B*, double stage regulator; *C*, low pressure regulator; *D*, Y-connection; *E*, constant temperature booth; *F*, skin cell; *G*, differential flowmeters; *H*, d.-c. Wheatstone bridges; *J*, timing mechanism and changeover circuit; *K*, single point recorder; *R*, electrolytic cells.

The Bridge.—A d. c. Wheatstone bridge was found satisfactory for measuring the decrease in resistance resulting from the addition of water to an electrolytic cell. The voltage was supplied by four, $1^1/_2$ volt dry cell batteries connected in series. The batteries and the detector were interchanged (7) to enable a more rapid standardization of the bridge. A variable resistor, in series with the batteries, was used to regulate the current flow and prevent overloading of the circuit as well as controlling the sensitivity of the bridge to water. The known resistances were a L-N AC-DC decade resistance box (No. 4754)[1] and a L-N slidewire box (No. 4261).[1] A rubber stopper was found suitable to hold the electrodes and the gas inlet and outlet tubes.

The gas tubes were made from 4 mm. i. d. Pyrex glass tubing and the electrodes were constructed by sealing 24 gauge platinum wire into 4 mm. i. d. Pyrex glass tubing so that 5 cm. of the wire extended out from the point of sealing. A 3-cm. portion extended inside the tube and was joined to the bridge through a mercury pool-type contact. The exposed 5-cm. length was wound in a tight, flat coil of about 1 cm. in diameter. The optimum distance between the electrodes was found to be 5 mm. The gas inlet tube was placed equidistant from both electrodes with the orifice turned away from the electrodes. Since in this study it was only necessary to determine the change in the resistance of the solvent-electrolyte system, the cell constant was not determined.

A number of solvent-electrolyte systems, previously reported, were studied using this bridge circuit and a L-N millivolt recorder (Speedomax type G model S, 60000 Series)[1] as the indicating device (1–4, 11–16). The systems were prepared in varying proportions and carefully tested for electrical response to 0.010-ml. aliquots of water added directly to the cell which was maintained at 21.0 ± 0.1° in a water bath. Each electrolyte was carefully dried prior to testing.

The Gaseous System.—High purity dry nitrogen gas[2] carried water vapor (perspiration) from a vapor cell or a skin cell to the electrolytic cell and, at the same time, stirred the mixture. All of the connections in the gaseous system were of either Pyrex glass or Tygon tubing.[3] Rubber was avoided since it has a tendency to absorb moisture. The pressure was regulated by a double stage tank gauge and a low pressure "pancake" type diaphragm valve.[4]

In the initial work only one bridge was standardized at a time; therefore, during the standardization procedure only one arm of the gaseous system was used. In the complete dual system the gas pressure was adjusted in either arm of the gaseous system by the stopcocks in the arms of the Y-connection. Minor evaporation of the electrolytic solvents was noted but this did not affect the results.

A vapor and a skin cell were made to enclose a given volume of water or area of skin from methyl methacrylate plastic.[5] The vapor cell contained a single groove 7 cm. long, 0.5 cm. wide, and 0.5 cm. deep, with suitable inlet and outlet orifices. The skin cell was of similar construction but consisted of two grooves which were intended to enclose two adjacent areas 1 cm. apart. A second plate was used to seal the skin cell when it was not enclosing an area of skin. In the vapor cell the second plate had a similar groove which represented the area of the skin covered. The cells were made airtight by applying a thin layer of petrolatum to the contact surface of the bottom plate or by applying mild pressure to the cell resting on the surface of the body.

The Dual Electrical Circuit.—Figure 2 shows the dual electrical system used to study a treated and an untreated area of the skin simultaneously. There are two d. c. Wheatstone bridges connected through a double pole, double throw (DPDT) relay[6] to the single point recorder. The source of power in each bridge was four $1^1/_2$ volt dry cell batteries. The bridges differed only in that a second slidewire box was not available, so two decade resistance boxes were connected in parallel and served the same purpose in the second bridge. The bridges were wired with No. 18 gauge, single strand wire (No. 8945)[7] and the leads from the DPDT relay were shielded wire (No. 8410)[7] because of the distance between the relay and the recorder. A DPDT relay was used as an inexpensive means of converting the single point recorder to a multipoint unit. The activating circuit of the DPDT relay consisted of a 45 volt dry cell battery connected in series with a momentary contact switch, *Km*. The momentary contact switch was activated by a cam drive which was connected to a small electrical motor through a pulley drive mechanism. The cam had a speed of 2 r. p. m. and was cut in such a manner as to activate the DPDT relay for two-thirds of its cycle. This causes one bridge to record for one-third of the cycle of the cam and the other bridge for two-thirds of the

[1] Leeds-Northrup Co., Philadelphia, Pa.

[2] Linde Air Products Co., Indianapolis, Ind.
[3] The United States Stoneware Co., Akron, Ohio.
[4] Regulator No. 70, The Matheson Co., Joliet, Ill.
[5] Rohm and Haas Co., Philadelphia, Pa.
[6] Type LM11, Potter and Brumfield Co., Princeton, Ind.
[7] Belden Wire Co., Chicago, Ill.

TABLE I.—A COMPARISON OF THE VOLUME OF WATER COLLECTED FROM THE VAPOR CELL TO THAT INDICATED BY A CHANGE IN THE RESISTANCE OF THE ELECTROLYTIC CELL.

Electrolytic Cell R_1				Electrolytic Cell R'_1			
		Microliters				Microliters	
Time[a]	mv.[b]	OV[c]	CV[d]	Time[a]	mv.[b]	OV[c]	CV[d]
60	0.40	30.0	33.0	30	0.90	30.0	39.0
60	0.39	30.0	32.0	30	0.44	20.0	19.0
60	0.64	40.0	43.0	32	0.93	28.0	41.0
45	0.39	30.0	32.0	30	0.77	25.0	34.0
30	0.32	30.0	28.0	30	0.59	28.0	26.0
30	0.30	30.0	25.0	30	0.91	28.0	40.0
50	0.52	30.0	43.0	30	0.62	29.0	27.0
60	0.52	30.0	42.0	45	0.78	40.0	34.0
45	0.31	35.0	26.0	30	0.83	30.0	36.0
30	0.54	30.0	45.0	30	0.75	28.0	33.0
30	0.47	25.0	39.0	30	0.62	22.0	27.0
				30	0.96	28.0	42.0
				34	1.05	30.0	46.0
Σ		340	388			366	444
μ'		30.9	35.3			28.2	34.2
σ'		3.75	7.30			4.50	7.70
μ_D[e]		33.1				31.2	
σ_D[f]		2.51				2.47	

[a] Time in minutes.
[b] Millivolts response per unit time.
[c] Observed value—the volume in μL. as indicated by a change in resistance of the recorder.
[d] Calculated value—the volume in μL. as determined by the loss in weight of the vapor cell, converted to volume.

[e] Mean: $n\mu = \sum_{j=1}^{n} Xj$

[f] Standard deviation: $(n-1)\sigma^2 = \sum_{j=1}^{n} (Xj - \mu)^2$

[g] Difference of two means: $n\mu_D = \sum_{j=1}^{n} \mu j$

[h] Standard deviation of the difference of two means: $\sigma_D{}^2 = \sigma'^2 + \sigma''^2$

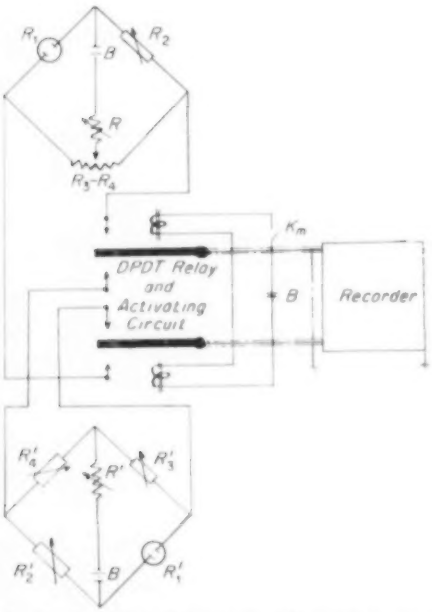

Fig. 2.—The dual Wheatstone bridge and change-over circuit.

cycle of the cam. This also supplied a means of differentiating between the two bridges and the two areas of skin, on one recording.

RESULTS

Electrolytes and Solvents.—Two electrolytes were studied with different solvents, NaCl A. R., which was dried at 140° for twenty-four hours and oxalic acid A. R., which was dried at 103° for twenty-four hours. The different solvent systems tested with a saturated solution of NaCl were methanol, methanol-benzene, methanol-dioxane, and methanol-acetone. The solvent systems used with oxalic acid were methanol, dioxane, methanol-dioxane, and methanol-acetone. In this case both the solvent and the electrolyte were varied extensively to obtain the most reproducible results.

The most effective solvent-electrolyte system was 25.0 ml. methanol (tech.), 5.0 ml. acetone N. F., and 1.0 Gm. oxalic acid. It was also found that the oxalic acid in the methanol-acetone mixture was not a critical factor, therefore, the amount of oxalic acid in solution could be varied by ±0.010 Gm. without appreciably affecting the results. Another advantage of oxalic acid was its complete solubility in the organic solvents in the concentrations used.

Standardization of the Electrolytic Cells.—Each cell-bridge combination had its own special properties which may not be identical with a similar combination, therefore, the two cells, R_1 and R'_1, were studied separately and a calibration curve was determined for each cell by adding 0.010-ml. (10λ) aliquots of water from a 10-λ pipet directly to the electrolytic cell. Figure 3 shows the response curves for each cell. Seven determinations were used to calculate each point on the response curves for R_1 and R'_1. The average responses for R_1 and R'_1 were 0.121 mv./0.010 ml. and 0.225 mv./ml., respectively, over the range of 0 to 0.150 ml. of H_2O. The reason

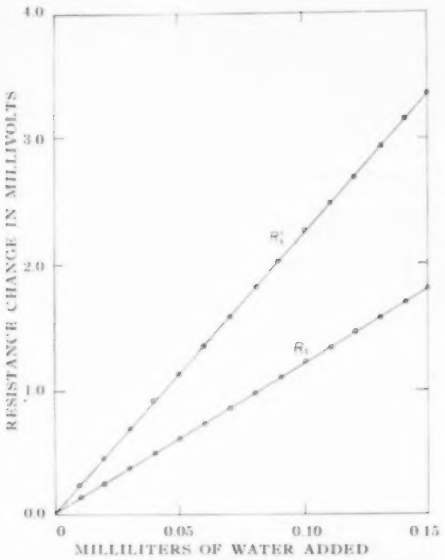

Fig. 3.—The relationship between the resistance change and 0.010 ml. per aliquot of water.

for the variation in these values was that the two bridges must standardize at the same time and maintain parallel baselines on the recorder for the two tests to be conducted simultaneously.

Comparison of the Vapor Cell with an Electrolytic Cell.—To prove that the procedure developed in this study was an analytical method of measuring moisture in gases, it was necessary to develop a cell in which known weights of water could be placed and the loss of weight (water) determined as a stream of gas passed over the water. Table I is a comparison of the loss of weight of the water sample with the amount of water absorbed by the electrolytic solution, as indicated by the change in resistance of the bridge. The temperature surrounding the vapor cell was 40.0 ± 1.0°. The weight of water was converted to volume.

SUMMARY AND CONCLUSIONS

1. A conductance method has been demonstrated for the measurement of water vapor in gases and is intended to be applied to the determination of the rate of perspiration flow from treated and untreated areas of the body.

2. Several combinations of solvent-electrolyte systems were screened for use in this investigation and methanol (tech.), 25.0 ml.; acetone N. F. 5.0 ml.; and oxalic acid A. R., 1.0 Gm. was selected as providing the most reproducible results.

3. Using the principles of the method developed, a dual system involving a dual bridge circuit was constructed which, in combination with a specifically designed cell, allowed two areas or samples to be studied continuously and simultaneously.

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An Evaluation of the Ability of Antiperspirant Compounds to Reduce Perspiration Flow*

By WILLIAM J. O'MALLEY† and JOHN E. CHRISTIAN

The objective of this study was to apply a previously developed electro-conductivity method of measuring moisture in gases and vapors to the determination of the ability of an antiperspirant compound to reduce perspiration flow. A skin cell was developed which enclosed two adjacent areas of skin. The optimum site of application of the skin cell was the forearm and the optimum environmental temperature of the subject was 35°. Three types of commercial bases were studied to determine their effect on the perspiration flow. Then six different commercial products and one compound not commercially available were compared to 24 per cent $Al_2(SO_4)_3 \cdot 18$ H_2O solution to show their effectiveness at reducing perspiration flow.

AN ELECTRO-CONDUCTIVITY method previously developed for measuring moisture in gases and vapors (1) was applied to the measurement of the effectiveness of several antiperspirant compounds to reduce sensible perspiration flow from a restricted area of the body.

Most of the previous methods used did not require a precise knowledge of the temperatures producing insensible perspiration, sensible perspiration, and profuse local perspiration, since they were *in vitro* methods. These methods can be divided between those which precipitate proteins (2, 3, 4) and those which adsorb water on an insoluble material (5–8). There were only two methods which simulated air currents, Palmes (9) used infrared analysis, and Hill and Hargood-Ash (10) measured the thermal conductivity of water vapor. In both of these cases a gas was passed over an enclosed area of the skin.

Using the electro-conductivity method it was necessary to determine the optimum temperature at which sensible perspiration was eliminated through the subject's sweat glands and ducts (11) but at which profuse local perspiring does not occur, and an optimum site of application of a skin cell.

A temperature of 35° was selected since it was sufficiently below the temperature necessary to stimulate profuse local sweating in those areas containing apocrine glands (12). The rate of profuse local sweating was such that it exceeded the ability of the gaseous system to evaporate the perspired water. The forearm was selected as the site of application of the skin cell since it was easily accessible in both men and women.

After having determined the optimum temperature and site of application of the skin cell, it was necessary to show that the amount of water collected from two adjacent areas at the same time was equal. Then three types of bases were studied to prove that the commercial bases did not affect the antiperspirant activity of the compounds, and finally, six different commercial products and one compound not commercially available were compared with 24 per cent w/w $Al_2(SO_4)_3 \cdot 18\ H_2O$ solution.

EXPERIMENTAL

Apparatus.—The preparation of the electrolytic cells and the apparatus was previously described (1).

Preparation of the Chemical Standard.—It was advisable to select a standard of comparison which would be easily obtainable, one which had been used before (5), and one which could be used at a later date with confidence that there were no hidden elements to affect the results. For these reasons $Al_2(SO_4)_3 \cdot 18\ H_2O$ was used in the form of a 24% w/w solution. The aluminum sulfate (24.0 Gm.) was dissolved in 75.8 Gm. of distilled water and 0.2 Gm. of Tween 20 to make 100 Gm. of a 24% w/w solution.

Preparation of Treated Areas of the Forearm.—The forearm was divided into a front and a back area. Each of these areas was then divided into two parts longitudinally, and the separate areas were marked off with a solution of silver nitrate. To the left side, in each case, was applied 0.07 ml. of aluminum sulfate solution. The other side went untreated or was treated with 90 mg. of cream or stick, or 0.07 ml. of lotion. The bases and the finished products (creams, sticks, and lotions) were representative commercial products. The solution of aluminum sulfate, the commercial bases and products, and the research compound were applied once each day for the duration of the test. The areas were washed each morning with a mild facial soap.

RESULTS

The Determination of Sites of Application.—There are three types of stimuli which cause perspiring in man; thermal, mental, and a combination of thermal and mental. These three types of stimuli produce perspiration at different areas on the body, and since the factors which affect mental perspiring are so varied from one individual to another, the areas of mental perspiring should be avoided when

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testing antiperspirants, even though some of these areas are common points of antiperspirant application (axillae). A broad surface which was easily accessible to both men and women was the forearm.

The Determination of the External Temperature. It was found the results were very erratic if only a portion of the body was submitted to a higher temperature; therefore, a special constant temperature booth was constructed for use during the experiment. Experiments were run at 25, 35, 40, and 45° ± 1°. The data obtained at 35° are shown in Table I. The ratio is of the left area to the right area under the skin cell, and the test time was fifteen minutes.

TABLE I.—A COMPARISON OF PERSPIRATION FLOW FROM LIMITED ADJACENT AREAS OF SKIN AT 35°

	Left[a]	Right[a]	Ratio[b]
	8.0	11.0	0.73
	12.0	10.0	1.24
	17.0	16.0	1.09
	14.0	15.0	0.93
	11.0	11.0	1.00
	8.0	8.0	1.00
	16.0	16.0	1.00
	14.0	16.0	0.88
	15.0	13.0	1.15
	17.0	15.0	1.13
	6.0	7.0	0.91
	12.0	13.0	0.94
	11.0	12.0	0.93
Σ	161.0	163.0	12.930
μ[c]	12.38	12.53	0.994
σ[d]	3.73	2.92	0.2135

[a] The number of μL. of water collected in fifteen minutes.
[b] Ratio of left to right.
[c] Mean: $n\mu = \sum_{j=1}^{n} Xj$, where $n = 13$.
[d] Standard deviation: $(n-1)\sigma^2 = \sum_{j=1}^{n} (Xj - \mu)^2$.

At 35° the atmosphere was sufficiently warm to induce a uniform flow of sensible perspiration from adjacent areas of the body (ratio = 0.994). Above this temperature the severe heat produced such unwanted effects as emotional unrest and excessive perspiration flow which the gas was not able to carry away, while below this temperature the amount of perspiration released was not enough to indicate reproducible results from adjacent areas.

The Comparison of Normal Skin with 24% w/w $Al_2(SO_4)_3 \cdot 18\ H_2O$ Solution.—Table II shows the microliters of water collected from two adjacent areas of the forearm and indicates the ratio of water collected from normal and aluminum sulfate-treated skin. The average value of the ratio of normal skin to aluminum sulfate-treated skin indicates that 1.94 times as much water was lost from normal skin as from aluminum sulfate-treated skin.

The Evaluation of Antiperspirant Bases.—Three types of commercial bases and normal skin were compared with 24% w/w $Al_2(SO_4)_3 \cdot 18\ H_2O$ solution. Table III indicates that approximately the same amount of water was collected from normal skin as was obtained from normal skin treated with a stick base, a cream base, and a lotion base. The results also indicate that the ratio between normal skin and aluminum sulfate-treated skin was similar to the ratio between the areas treated with the three bases and aluminum sulfate. One test was run once a day for three days on each area; an area being a treated or a controlled portion of skin adjacent to each other.

TABLE II.—A COMPARISON OF THE AMOUNTS OF WATER COLLECTED FROM A LIMITED AREA OF NORMAL AND 24% w/w ALUMINUM SULFATE-TREATED SKIN

	Normal	Aluminum Sulfate	Ratio[a]
	11.5	6.7	1.72
	8.3	5.9	1.41
	15.6	10.1	1.54
	6.6	4.2	1.57
	11.5	4.2	2.74
	9.3	5.0	1.86
	12.5	5.0	2.25
	11.1	5.9	1.88
	16.0	7.6	2.11
	9.7	4.2	2.31
Σ	112.1	58.8	19.39
μ	11.21	5.88	1.939
σ	2.56	1.87	0.413

[a] The ratio of the volume of water collected in fifteen minutes from normal skin to aluminum sulfate treated skin in microliters.

TABLE III.—A COMPARISON OF THE AMOUNT OF WATER COLLECTED FROM A LIMITED AREA OF SKIN TREATED WITH ANTIPERSPIRANT BASES

Normal Skin or Base	Microliters H_2O[a] Normal or Base	Aluminum Sulfate	Ratio[b]
Normal	13.0	8.0	1.625
Stick	13.3	8.3	1.602
Cream	12.0	8.0	1.500
Lotion	12.0	7.5	1.600

[a] Each value represents the mean of three experiments on the same limited area.
[b] The ratio of the means of normal skin or areas treated with the three bases to adjacent aluminum sulfate treated areas.

Constant Values.—It was noted that as each of the products was applied on successive days, the variation in the amount of water perspired tended to decrease, and that after a certain length of time (four to six days for the products, three to four days for aluminum sulfate) a fairly constant volume and ratio were obtained (see Table IV). Variations in the total volume changed with the temperature and humidity, but the ratio remained fairly constant.

Commercial Antiperspirant Products.—Six commercial and one research product were compared

TABLE IV.—A COMPARISON OF THE PERSPIRATION REDUCING PROPERTIES OF ALUMINUM SULFAMATE CREAM WITH 24% w/w ALUMINUM SULFATE SOLUTION OVER A LIMITED AREA OF SKIN

Days	Microliter of H_2O, μL.[a] Aluminum Sulfamate	Aluminum Sulfate	Ratio[b]
1	6.1	4.6	1.34
2	5.9	4.2	1.41
3	7.3	5.0	1.45
Σ	19.3	13.8	8.40
μ	6.42	4.60	1.400
σ	0.682	0.438	0.101

[a] Each value represents the mean amount of water collected from the same area twice daily; a constant volume of water was collected after four days.
[b] The ratio of the means of aluminum sulfamate to aluminum sulfate-treated skin.

TABLE V.—A COMPARISON OF THE AMOUNT OF SENSIBLE PERSPIRATION COLLECTED FROM LIMITED SKIN AREAS TREATED WITH ANTIPERSPIRANT PREPARATIONS AND 24% w/w $Al_2(SO_4)_3 \cdot 18H_2O$ SOLUTION

Antiperspirant Preparations	Mean Response[a] μL.[b]	$Al_2(SO_4)_3 \cdot 18H_2O$ Soln. Mean Response[a] μL.[b]	Mean Ratio
A[c]	8.4	5.9	1.52[d]
B	8.8	5.3	1.68
C	9.2	4.9	2.09
D[e]	5.6	4.6	1.21
E	6.7	4.9	1.40
F	7.4	4.9	1.52
G	6.4	4.6	1.40

[a] A mean of ten determinations on five consecutive days were used to calculate the mean response of each antiperspirant preparation tested.

[b] The microliters of water collected in fifteen minutes were calculated using the standard curves for electrolytic cells R_1 and R'_1.

[c] Active ingredients: A, aluminum hydroxide gel, B, aluminum chloride and formaldehyde, C, aluminum chlorohydroxide complex, aluminum chloride, and formaldehyde, D, aluminum methionate, E, sodium aluminum lactate and hexachlorophene, F, aluminum chlorohydrates, G, aluminum sulfamate.

[d] This ratio indicates that 1.52 times as much sensible perspiration was collected from the skin areas treated with antiperspirant preparation A as that from the aluminum sulfate-treated area.

[e] A preparation prepared by J. E. Haberle, M.S. thesis, Purdue University, Lafayette, Ind., June, 1958.

with the aluminum sulfate solution. The antiperspirants were applied to the chosen areas until constant volumes were obtained, and then the experiment was continued for another three days. Table IV is an example of the type of data obtained. The active ingredient was aluminum sulfamate in a cream base.

Table V shows the mean volumes of water collected when the six commercial products and the one research product (aluminum methionate) were compared with 24% w/w aluminum sulfate solution. The volume ratio indicates the ability of the antiperspirant to reduce perspiration flow as compared with 24% w/w aluminum sulfate solution. The results indicate that the research compound, aluminum methionate, was the most effective of the products studied to reduce sensible perspiration flow.

SUMMARY AND CONCLUSIONS

1. A continuous recording *in vivo* method of measuring sensible perspiration was used to evaluate the relative perspiration properties of six commercial and one research antiperspirant preparation in comparison with 24% w/w $Al_2(SO_4)_3 \cdot 18\ H_2O$ solution. The results indicated that aluminum methionate was the most effective agent for reducing perspiration flow from the treated area.

2. The method was also used to evaluate three different types of antiperspirant bases: the stick, the cream, and the lotion. The results when compared with normal skin indicated that the bases had no effect on the antiperspirant action of the preparations.

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Determination of Vitamin A: Errata*

Sir:

In the paper titled "A Simplified Procedure for the Determination of Vitamin A" (1), the first footnote carried under Table I is incorrect. This should read as follows:

$$f(\text{MS}) = \frac{A_{\text{corr.}}}{A_{\text{uncorr.}}}$$

$$A_{\text{corr.}} = 6.815\,(A_{328}) - 2.555(A_{312}) - 4.260(A_{334})$$

$$A_{\text{uncorr.}} = A_{328}$$

Furthermore, in all Tables, F(MS) should read f(MS).

(1) Napoli, J. A., Senkowski, B. Z., and Motchane, A. E., THIS JOURNAL, **48,** 611 (1959).

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Notes

A Note on Esters of β-Phenyl-α-benzyloximinopropionic Acid*

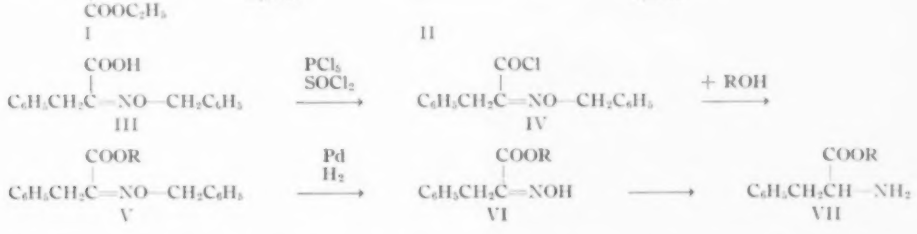
By LOUIS GASS† and JOHN W. MARTIN, Jr.

Selected esters were prepared for the purpose of studying the debenzylation reactions when the alcohol portion of the esters was modified. A method is given to obtain esters of oximino acids. Except for phenolic derivatives, the esters were fairly easily prepared from the alcohol and the acid chloride.

IN A REPORTED synthesis of amides of α-amino acids (1) the α-benzyloximino acid is the key intermediate. In the present investigation esters of α-benzyloximino acid have been synthesized (V). These are intermediates necessary in the study of the hydrogenolysis and reduction reactions leading to esters of α-amino acids (VII). The entire sequence of reactions is shown below. Compounds of type VI and VII are not reported in this paper.

The esters thus prepared represent different categories which can be studied chemically and pharmacologically. From preliminary experiments with the debenzylation reaction (V to VI) it appears that the rate of debenzylation is greatly influenced by the type of derivative.

Compound I, a substituted malonic ester, was prepared by a standard procedure (2). The nitrosation of I was done according to the method of Waters and Hartung (3), with a slight modification using isopropyl nitrite rather than the butyl nitrite. Compound II was obtained in good yields. Efforts to prepare the acid chloride directly proved unsuccessful (4) so compounds III and IV were prepared by methods reported by Hartung and coworkers (1–5). Compound V, heretofore not prepared by this route, involved the reaction of the acid chloride directly with the alcohol in absolute ether.



EXPERIMENTAL

All melting points reported were obtained by use of a Fisher-Johns melting point apparatus.

Amyl β-Phenyl-α-benzyloximinopropionate.—Four and four-tenths grams (0.05 *M*) of freshly distilled amyl alcohol was placed in a three-necked round-bottom flask to which was added 60 ml. of dry ether. The flask was placed in an ice bath and, with mechanical stirring, an equimolar portion of a benzene solution of the acid chloride was dropped in slowly. After the complete addition of the acid chloride stirring was continued for ten minutes. The yellow solution was washed with 100 ml. of a saturated solution of sodium bicarbonate and then dried over calcium chloride for forty-eight hours. After removal of the ether under vacuum there remained 16.9 Gm. (quantitative) of a yellow viscous oil. Upon attempted vacuum distillation the product decomposed.

Anal.—Calcd. for $C_{21}H_{25}NO_5$: N, 4.13; sapon. equiv., 339. Found: N, 3.95, 3.91; sapon. equiv., 338.2, 339.6.

Benzyl β-Phenyl-α-benzyloximinopropionate.—Ten and eight-tenths grams (0.1*M*) of freshly distilled benzyl alcohol was dissolved in 50 ml. of dry ether in a small beaker cooled in an ice bath. To this was added slowly, with rapid stirring, a benzene solution of an equimolar amount of acid chloride, prepared from β-phenyl-α-benzyloximino propionic acid. After standing for about ten minutes in the ice bath the ether was removed and the remaining solid was recrystallized twice from alcohol with the addition of Nuchar. After cooling, the crystals were collected. After drying *in vacuo* over P_2O_5 the product weighed 31.5 Gm. (87.7%), m. p. 79–80°.

Anal.—Calcd. for $C_{23}H_{23}NO_5$: N 3.90; sapon. equiv., 359. Found: N, 3.78, 3.72; sapon. equiv., 361, 358.1, 358.8.

Cyclohexyl β-Phenyl-α-benzyloximinopropionate.—Essentially the same procedure was used as for the amyl derivative. From 5 Gm. (0.05*M*) of

* Received April 13, 1956, from Butler University, College of Pharmacy, Indianapolis 7, Ind.
Abstracted from a thesis submitted as partial fulfillment of the requirement for the degree of Master of Science, Butler University, College of Pharmacy, Indianapolis, Ind.
Presented to the Scientific Section, A. PH. A., Detroit meeting, April 1956.
† Present address: Whitehal Laboratories, Hammonton, N. J.

cyclohexanol and an equimolar quantity of the acid chloride, 15.5 Gm. (88%) of liquid was obtained. The boiling point by the semimicro method was 189°, with decomposition.

Anal.—Calcd. for $C_{22}H_{25}NO_3$: N, 3.99; sapon. equiv., 351. Found: N, 3.89, 3.83; sapon. equiv., 350.7, 351.3.

Naphthyl β-Phenyl-α-benzyloximinopropionate.—Essentially the same procedure was used as for the amyl ester. From 7.2 Gm. (0.05 *M*) of purified α-naphthol and an equimolar amount of the acid chloride, 16.5 Gm. (83.5%) of a viscous oil was collected. Naphtha was used as the solvent. The oil decomposed upon attempted distillation.

Anal.—Calcd. for $C_{26}H_{21}NO_3$: N, 3.54; sapon. equiv., 395. Found: N, 3.36, 3.25; sapon. equiv., 389.8, 390.6, 389.6.

Furfuryl β-Phenyl-α-benzyloximinopropionate.—Nine and eight-tenths grams (0.1 *M*) of freshly distilled furfuryl alcohol was placed in a beaker cooled in an ice bath. Sixty milliliters of anhydrous ether and 7.9 Gm. (0.01 *M*) of freshly distilled pyridine were added and then, with rapid stirring, an equimolar portion of a benzene solution of the acid chloride. There was an immediate bubbling and the solution turned black. This mixture was allowed to stand in the ice bath for fifteen minutes and then was washed several times with water to remove the pyridine hydrochloride. After evaporation of the ether and long standing the mixture crystallized. The large crystals were purified by recrystallization from alcohol and water. After drying *in vacuo* over P_2O_5 the product weighed 21.5 Gm. (61.6%), m. p. 38–39°.

Anal.—Calcd. for $C_{19}H_{19}NO_4$: N, 4.01; sapon. equiv., 349. Found: N, 3.85, 3.90, 3.84; sapon. equiv., 347.5, 347.6.

SUMMARY

Five selected esters of β-phenyl-α-benzyloximinopropionic acid have been prepared and characterized.

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A Note on the Synthesis of C-14 Carboxyl Salicylic Acid by the Halogen-Metal Interconversion Reaction*

By WILLIAM F. BOUSQUET and JOHN E. CHRISTIAN

IN ORDER TO CARRY OUT a study of the central nervous system distribution and metabolism of C-14 carbonyl salicylamide in the rat (1), it was necessary to prepare C-14 carboxyl labeled salicylic acid for use in synthesizing the amide.

The preparation of C-14 carboxyl salicylic acid has been described in the literature by a number of authors. Mandel and Smith (2), Schayer (3), and Borst and Christian (4) have described the synthesis of this labeled compound by the Kolbe-Schmitt reaction. Medenwald and Haberland (5) have recently described a synthesis of carboxyl labeled salicylic acid by the Grignard reaction.

The method of synthesis chosen was the halogen-metal interconversion reaction on *o*-bromoanisole followed by carbonation of the lithium salt *in vacuo* with $C^{14}O_2$. The C-14 carboxyl *o*-methoxybenzoic acid so prepared was then treated with HI to liberate salicylic acid.

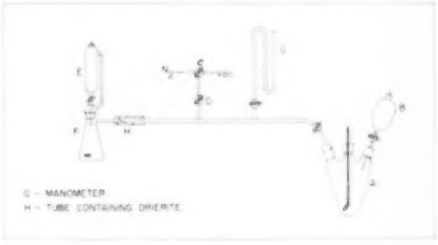
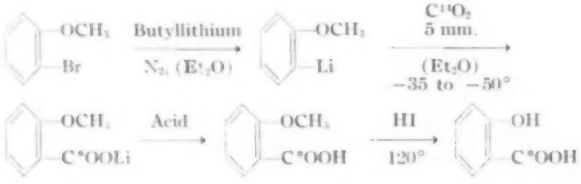


Fig. 1.—Gaseous carbonation apparatus.



The primary advantages of this method in the synthesis of C-14 carboxyl salicylic acid are that it is not necessary to work with $C^{14}O_2$ under heat and pressure, and specificity of labeling is attained. Furthermore, C-14 carboxyl *p*-hydroxybenzoic acid is not a by-product in the reaction as is the case with the Kolbe-Schmitt procedure.

* Received August 21, 1959, from the Bionucleonics Department, Purdue University, Lafayette, Ind.

The synthesis was scaled to give a theoretical yield of 1.0 Gm. (0.00725 M) of salicylic acid. The apparatus used in the carbonation, similar to that of Dauben, *et al.* (6), and Rockerbie (7), is shown in Fig. 1. Seven runs were made without radioactivity in order to perfect the synthesis. Four additional runs were made using small quantities (e. g., 100–200 μc.) of radioactivity. Yields of purified salicylic acid ranged from 6 to 43% of the theoretical. The procedure described below is that followed in the final run with 25 mc. of C-14 activity.

PROCEDURE

The *n*-butyllithium was prepared by the procedure of Gilman, *et al.* (8), and assayed before use by the method of Gilman and Haubein (9). All glassware used in the synthesis was cleaned with chromic acid-sulfuric acid cleaning solution, rinsed with distilled water, oven-baked, and flushed with dry nitrogen immediately before use.

A measured amount of the *n*-butyllithium solution in ether corresponding to 20 mM was transferred to reaction flask (*A*) while the system was being flushed with dry nitrogen. The nitrogen was turned off, stopcock (*D*) closed, and addition funnel (*B*) containing 1.95 Gm. (1.3 ml., 10 mM) of *o*-bromoanisole in 5 ml. of anhydrous ether set in place. A Dewar flask containing acetone-dry ice was placed under the reaction flask. The *o*-bromoanisole was rapidly added to the butyllithium solution and the reaction allowed to proceed with gentle stirring for fifteen minutes. The sitrrer was shut off and the acetone-dry ice bath replaced with one of liquid nitrogen. The contents of the reaction flask were frozen solid and the system evacuated to about 3 mm. The acetone-dry ice bath was again set in place. When the pressure in the system remained constant at from 5 to 8 mm. the stirrer was set at high speed and concentrated sulfuric acid added from addition funnel (*E*) to the $BaC^{14}O_3$ in generator flask (*F*).

The magnetic stirrer in the generator flask was set in motion and stirring continued until gas evolution appeared complete. After an initial rise, the pressure in the system fell rapidly as the carbon dioxide was taken up in the reaction mixture. Stirring was continued until the pressure remained constant. The contents of the reaction flask were alternately frozen solid and thawed twice to insure maximum uptake of $C^{14}O_2$.

The male stopper in addition funnel (*B*) was replaced with a T/S 24/40 connecting tube, and a 12-inch length of rubber tubing used to connect this to a barium hydroxide absorption train. The system was then opened to allow nitrogen to enter and bring the system to atmospheric pressure. Stopcock (*C*) was opened and nitrogen flushed through the system into the absorption train to allow collection of unreacted $C^{14}O_2$. Flushing was continued for twenty-five minutes. The nitrogen was then shut off, the system closed, and the absorption train removed.

Ten milliliters of a 20% solution of hydrochloric acid was placed in addition funnel (*B*) and added, with rapid stirring, to the reaction mixture. The contents of flask (*A*) were then transferred to a 125-ml. separatory funnel. The flask was washed with two 10-ml. portions of ether and distilled water, and the washings added to the separatory funnel. The aqueous layer was drawn off and set aside. The ether layer was extracted with three 5-ml. portions of 10% sodium hydroxide solution. The aqueous layer was extracted with three 5-ml. portions of ether, and this was extracted with two 5-ml. portions of 10% sodium hydroxide. The sodium hydroxide extracts were pooled, set in an ice bath, and concentrated hydrochloric acid added dropwise to precipitate C-14 carboxyl *o*-methoxybenzoic acid. The acid was collected by suction filtration and oven dried at 60°.

The dried crystals obtained above were transferred to a 50-ml. round-bottom flask to which was added 5 ml. of 47% hydriodic acid (sp. gr. 1.50). The flask was fitted with a reflux condenser, set in an oil bath, and the temperature raised and maintained at 120–125° for thirty minutes. Upon cooling, long white needles of C-14 carboxyl salicylic acid precipitated. The mixture was allowed to stand overnight and the salicylic acid then removed by suction filtration. The crystals were dried at 110° and allowed to stand exposed to air for forty-eight hours to remove any adsorbed iodine.

Pure salicylic acid recovered amounted to 67.0 mg. with a specific activity of 5.59×10^6 dpm/mg. An additional 13.0 mg. of material was recovered, having a specific activity of 3.52×10^6 dpm/mg. The chemical yield of purified C-14 carboxyl salicylic acid was 6.7%. Radiochemical yield[1] was 8.2%. A melting point was not taken on this material due to its high activity. However, melting points were determined on the salicylic acid prepared in the dry runs and these were in agreement with published values for salicylic acid.

Chemical and radiochemical purities of the labeled compound were determined using a combination of paper chromatography and autoradiography. Chromatograms were prepared using the ascending technique with a solvent system of benzene:glacial acetic acid:water (4:4:2). Whatman No. 1 paper was used. Color development was attained by spraying with ferric nitrate solution. The R_f value of the synthesized compound was found to be identical with that for reference salicylic acid.

Preparation of an autoradiogram from the chromatogram using Eastman-Kodak "No-Screen" X-ray film showed the presence of but one spot corresponding to the synthesized material. No radiochemical contaminants were present.

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[1] The specific activity of the synthesized material was determined by dissolving 0.5065 mg., accurately weighed, in 50 ml. of toluene containing 50 mg. of carrier salicylic acid. The activity of 100 λ aliquots of this solution was determined using the Tri-Carb liquid scintillation spectrometer. Correction for counter efficiency was made using a National Bureau of Standards C-14 carboxyl benzoic acid beta ray standard.

A Note on Improvement of Separations in Paper Partition Chromatography*

By JERE E. GOYAN

FOR MANY YEARS it has been recognized that the separation of two substances by multiple fractional extraction is greatest when the volume ratio of the two solvents (Vx/Vy) is equal to $\sqrt{1/\alpha_1 \alpha_2}$, where α_1 and α_2 are the partition coefficients for the two components being separated (α_1 = mg. 1 per cc. of x/mg. 1 per cc. of y) (1). The same principles are involved in paper chromatography, which may be approximated as a series of extractions, and it might be expected that a similar ratio would be important in partition chromatography. In the course of a project on the separation of barbiturate isomers such a relationship was found.

The operational definition of R_f may be stated as

$$R_f = A_M/(A_M + \alpha A_S) \qquad \text{(Eq. 1)}$$

(2) where A_M is the cross sectional area of the mobile phase, A_S is the cross sectional area of the stationary phase, and α is the partition coefficient for the component between the mobile and stationary phase.

Solving Eq. 1 for α one obtains

$$\alpha = \frac{A_M}{A_S}\left[\frac{1}{R_f} - 1\right] \qquad \text{(Eq. 2)}$$

then defining $\beta = \alpha_1/\alpha_2$

$$\beta = R_{f2}(1 - R_{f1})/R_{f1}(1 - R_{f2}) \qquad \text{(Eq. 3)}$$

The most desirable separation for two components is when $R_{f2} - R_{f1}$ is a maximum. Solving Eq. 3 for R_{f2} and subtracting R_{f1} from both sides of the equation, one obtains

$$y = R_{f2} - R_{f1} = \beta R_{f1}/(1 - R_{f1} + \beta R_{f1}) - R_{f1} \qquad \text{(Eq. 4)}$$

Then setting the first derivative of y with respect to R_{f1} equal to zero and solving for R_{f1} the following equation is found

$$R_{f1} = 1/(1 + \sqrt{\beta}) \qquad \text{(Eq. 5)}$$

Rewriting Eq. 1 in terms of volumes gives (3)

$$R_{f1} = V_M/(V_M + \alpha V_S) \qquad \text{(Eq. 6)}$$

Then equating 5 and 6 and solving one obtains

$$V_S/V_M = \sqrt{1/\alpha_1\alpha_2} \qquad \text{(Eq. 7)}$$

which is the usual equation for maximizing separation in multiple fractional extraction procedures. This shows the correlation which is to be expected if paper partition chromatography can be approximated as a multiple extraction process.

Unfortunately, adjustment of the volumes of the stationary and mobile phases is difficult. It is possible to try several different papers and to choose the one giving the best separation. There is, however, another method of adjustment of R_{f1} in cases where three component systems are used. When small changes are made in phase concentrations β changes very little, since both values of α change in the same direction. Thus it becomes possible to change R_{f1} considerably while having only secondary effects on β.

These principles may be illustrated by some experimental data on the amino acids, glycine and alanine. Block, *et al.* (4), give data for these two amino acids in two different butanol:acetic acid:water systems, these data contributing the first two rows of Table I.

TABLE I.—R_f VALUES OF GLYCINE AND ALANINE IN BUTANOL:ACETIC ACID:WATER MIXTURES

Acetic Acid, % v/v	Glycine	Alanine	β
5[a]	0.03	0.05	1.9
10.6[a]	0.05	0.09	1.7
12.5	0.25	0.33	1.5
13.0	0.33	0.44	1.6

[a] Data from Block, *et al.* (4).

At first glance it might appear impossible to separate the two amino acids with this solvent system. However, solving for β using Eq. 3, gives values of 1.9 and 1.7. Solving for R_{f1} in Eq. 5 (using α = 1.8) gives a value of 0.43. Thus if would appear that better separations could be expected if R_{f1} is increased. In order to test this the following experiment was undertaken. The R_f should increase as the mobile phases become a better solvent for the amino acid, therefore the amount of acetic acid was increased. Values of 12.5 and 13.0% v/v acetic acid (equal parts of butanol and water) were chosen since greater percentages lead to a one phase system. These were run on Whatman No. 1 filter strips with descending formation. Table I shows the results of these experiments. Thus it was possible to get reasonable separations with a system which originally did not look promising. Parallel experiments using Whatman No. 4 and 54 paper gave R_{f1} values which were smaller and the separation was correspondingly less.

TABLE II.—R_f VALUES OF PENTOBARBITAL (I) AND ITS 1-ETHYLPROPYL ISOMER (II) IN BENZENE:10% ACETIC ACID:ETHANOL MIXTURES

Ethanol, % v/v	I	II	β
25	0.90	0.91	1.1
15	0.85	0.88	1.3
10	0.77	0.81	1.3
0	0.71	0.78	1.4

In the experiments, with pentobarbital and its 1-ethylpropyl isomer, using a benzene:10% acetic acid:ethanol system and reversed phase (strips treated with 5% SF-96 (300) silicone fluid, General Electric, in *n*-pentane and dried in an oven for three minutes at 150°) ascending development it was

* Received February 15, 1960, from the College of Pharmacy, University of Michigan, Ann Arbor.

This research was supported by a grant from the Horace H. Rackham School of Graduate Studies, The University of Michigan.

found that R_f was too large and that decreasing the ethanol concentration led to an improvement in the separation, as shown in Table II.

The general method may be extended, of course, to larger numbers of substances. Analysis of preliminary data in this manner should make it possible to make a more rational decision as to means of improving desired separations.

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Book Notices

New and Nonofficial Drugs 1960. Evaluated by A. M. A. Council on Drugs. Medical Dept., J. B. Lippincott Co., East Washington Square, Philadelphia 5, Pa., 1960. xxviii + 768 pp. 12.5 x 19 cm. Price $3.35.

The new subtitle for the 1960 edition of N. N. D. reads: "An annual compilation of available information on drugs, including their therapeutic, prophylactic, and diagnostic status, as evaluated by the Council on Drugs of the American Medical Association." Its scope comprises agents proposed for use whether or not their usefulness has been definitely established. The A. M. A. thus attempts to inform the physician about the values and dangers of new drugs as soon as responsible and authoritative opinions can be obtained. Forty-five new monographs have been added in N. N. D. 1960. It is to be hoped that the excellent work of the Council on Drugs could be broadened to publish its evaluations of new drugs even sooner, without sacrificing its unbiased, thorough approach. This might discourage the reading of hastily published opinions by other less qualified medical "experts."

The Merck Index of Chemicals and Drugs. 7th ed. Edited by PAUL G. STECHER, M. J. FINKEL, and O. H. SIEGMUND. Merck & Co., Inc., Rahway, N. J., 1960. xi + 1641 pp. 18 × 25 cm. Price $12.

This exceptionally useful and reliable reference book has 400 pages more than the 6th ed. of "The Merck Index," which was reviewed in THIS JOURNAL, **41**, 339(June 1952). The increase in page size also is significant, from 16 × 23.5 cm. in the 6th to 18 × 25 cm. in the 7th ed. The new edition includes nearly 10,000 descriptions of individual substances, more than 3,300 structural formulas, and about 30,000 names of chemicals and drugs in a separate, alphabetically arranged and cross-indexed listing. The separate listing should be utilized, rather than leafing through the extensive encyclopedic text, to find any substance or compound by its systematical-chemical, generic, common, brand, or trade name.

A special section lists more than 400 organic "name" reactions with a description and structural representation of each. Additional information includes a list of prescription notations and a table of isotonic solutions of drugs.

Pharmacopoea Internationalis, 1st Edition Supplement. World Health Organization, Geneva, 1959. Official distribution agent for the U. S. Columbia University Press, 2960 Broadway, New York 27, N. Y. xx + 224 pp. 16 x 24 cm. Price $5.

This supplementary volume containing 94 additional monographs and 17 appendixes completes the first edition of the International Pharmacopoeia (Ph. I.). Volumes I and II were reviewed in THIS JOURNAL, **41**, 222(1952) and **45**, 434(1956). The Ph. I. is a collection of recommended specifications which are not intended to have legal status, as such, in any country but which can serve as a basis for the establishment of national standards. Appendix 7, titled "Preparation of Isotonic Solutions," includes graphs showing the amount of sodium chloride (or potassium nitrate) to be added to solutions of 67 drugs to obtain isotonicity. A composite index for Volumes I and II and the Supplement is appended. A table comparing the titles and strength standards of drugs appearing in both Ph. I. and N. F. XI will be included in the General Information section of N. F. XI.

Year Book of Drug Therapy. 1959-1960 Series. Edited by HARRY BECKMAN. The Year Book Publishers, Inc., 200 East Illinois St., Chicago 11, Ill., 1960. lxxx + 570 pp. 13 x 19.5 cm. Price $8.50.

This edition of the Year Book continues Dr. Beckman's excellent coverage and condensation of the medical literature. He has added a section in which he gives brief opinions on new drugs for which FDA new drug applications became effective during the period from September 1958 to September 1959.

American Drug Index 1960. By CHARLES O. WILSON and TONY EVERETT JONES. J. B. Lippincott Co., East Washington Square, Philadelphia 5, Pa., 1960. 712 pp. 13.5 × 20.5 cm. Price $5.75.

The 1960 edition of "American Drug Index" brings up to date this very useful reference of pharmaceutical chemicals and preparations. The original format and concise style of coverage is unchanged.

The United States Pharmacopœial Convention, Inc.

ANNUAL FINANCIAL STATEMENT

Based upon the Report of the Auditor for the year ended December 31, 1959

STATEMENT OF INCOME AND EXPENSE

Income:			
Sales of Pharmacopeias:			
Collections		$25,133.25	
Add—Increase in accounts receivable		454.90	
			$ 25,588.15
Sales of reference standards:			
Collections		$49,742.00	
Add—Increase in accounts receivable, and adjustment o. prior years' collections		2,323.15	
			52,065.15
Interest on investments, less amortization of bond premiums of $22.65			6,839.85
Interest on savings deposits			6,012.43
Use of text by others			25.00
Miscellaneous			121.34
			$ 90,651.92
Expense:			
Revision		$86,330.41	
Less—Increase in inventory		340.76	
			$ 85,989.65
Administration			8,783.67
Publications		$27,034.43	
Add—Decrease in inventory		10,543.02	
			37,577.45
Headquarters		$ 5,658.97	
Add—Depreciation expense:			
Building	$2,430.77		
Furnishings and equipment	4,208.99	6,639.76	
			12,298.73
Convention			4,421.75
			$149,071.25
Excess of Expense over Income			$ 58,419.33

STATEMENT OF CASH RECEIPTS AND DISBURSEMENTS (GENERAL ACCOUNT)

Receipts:	
Sales of pharmacopeias	$ 25,133.25
Sales of reference standards	49,742.00
Interest on investments	6,862.50
Interest on savings deposits	6,695.86
Use of text by others	25.00
Miscellaneous	121.34
Transfer from savings accounts	18,000.00
	$106,579.95

	Revision	Administration	Publications	Headquarters	Convention	Furnishings and Equipment	Total
Disbursements:							
Printing and binding	...	...	$27,034.43	...	...	...	$ 27,034.43
Salaries	$57,209.72	$5,590.20	...	$1,794.20	$ 390.20	...	64,984.32
Meetings	2,613.77	1,455.42	...	...	3,596.78	...	7,665.97
Supplies	1,113.52	46.44	...	...	240.27	...	1,400.23
Postage and telegrams	1,709.02	36.11	...	...	194.50	...	1,939.63
Utilities	...	...	...	2,328.74	...	...	2,328.74
Repairs and maintenance	...	...	...	1,351.03	...	...	1,351.03
General	23,684.38*	1,655.50	...	...	...	...	25,339.88
Insurance and taxes	...	...	...	185.00	...	...	185.00
Furnishings and equipment	...	...	...	...	...	$478.75	478.75
	$86,330.41	$8,783.67	$27,034.43	$5,658.97	$4,421.75	$478.75	$132,707.98

* Research Assistants		$ 4,166
Reference Standards supplies		5,137
Retirement and Social Security		11,868
Promotion		103
Miscellaneous		2,410
		$23,684

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